

What future for Japan's universities?

Japan's universities are still urgently in need of the autonomy that would enable them to reform themselves; the ministry in charge of them should embrace that as its goal.

THE enviable social stability of Japan rests on the general confidence that there are rarely abrupt changes. Earthquakes apart, continuity over the decades is a goal in its own right. And it is seriously impolite to make a bargain with a person only to go back on it afterwards, as for example by changing the terms of a person's employment. Those are some of the reasons for the paradox that the national universities of what is arguably the richest and certainly the most productive country in the world are now so urgently in need of change. Last week's *Nature* conference in Tokyo (see page 721) uncovered the case for reform at the end of two days, perhaps because the Japanese could no longer bear the frustration of learning of the dynamic (if somewhat breathless) changes afoot among their neighbours in the Pacific. What is now required?

Elsewhere, it would seem simple. The ministry (called Monbusho) that covers running costs should not also be the chief source of academic research support. As everywhere else, Japan needs an independent research council operating strictly by independent judgements of which research deserves support. Then Japan's national universities need administrative autonomy, with the right to run their own academic businesses (and occasionally to make mistakes in doing so). They also need means of forcing coherence on their own faculties, winning consensus on common goals even from those who now interpret academic freedom as a licence to follow solitary aims (or none), and the habit of exposing individuals and groups to the rigours of objective assessment, in teaching and research.

To blame Monbusho (the Ministry of Education and Culture) is a natural temptation, and is justifiable. But the detail with which the finances of the universities are regulated by people whose allegiance is primarily to the ministry, not the university, is a consequence of Japan's view of the sanctity of public money. Ordinarily, only public servants are allowed to spend it, and then only by a meticulously devised rulebook. Departures from this principle can occasionally cause trouble (see, for example, *Nature* 372, 579–580; 1994), but the present intrusiveness of the administrators in Japan's universities thoroughly undermines their cohesiveness. The only good solution would be to reconstitute the national universities as public corporations. That would provide constitutional lawyers with a field-day, but no task is more urgent.

Many of Monbusho's officials are conscious of the need for change, and say so. But in the endless battle between

Tokyo's ministerial bureaucracies, to relinquish control of something is to seem weak. The time has long since passed when Monbusho can hope to win (or even keep) friends by sitting on its hands while the universities it is supposed to nurture are robbed of the effective autonomy they need. □

Chance makes good?

Britain's national lottery is a bad business, but research should make good use of it while it can.

BRITAIN'S month-old national lottery may be elective regressive taxation, taking money from those whose acquaintance with wealth is that they often dream of it (*Nature* 372, 304; 1994), but it has arrived and should be put to good use. One of its aims is to support good causes, notably charities, culture and the arts. There is some anxiety that this new flow of funds will let the government shrug off many of its present responsibilities, which is one good reason why the British research councils should not go looking for lottery funds. But there are deserving beneficiaries in the scientific world that need not be so choosy.

There is, for example, Burlington House, off London's Piccadilly, which houses (as well as the Royal Academy of Arts) a number of learned societies — the Linnean Society, the Royal Astronomical Society and the Royal Institute of Chemistry, for example. Hitherto, they have lived there rent-free, but they will be expected to bear the costs of maintaining the fabric of their buildings once the government devolves that responsibility to an independent central agency. These societies are charitable, right enough. Their cultural value is also self-evident. Were they so minded, they could make a good case for a rake-off from the lottery. They should not be deterred by the calculation that their interest in science makes them cultural pariahs.

Meanwhile, the lottery has run into controversy (in week three) over a request for anonymity by a man who won a 'jackpot' worth nearly £18 million. At one stage, the lottery organizers won an injunction from the courts to prevent the disclosure by the press of the man's name, but lost it the following day. Since then, there has been a great deal of humbug about press intrusion. But how are the operators, a company called Camelot, to demonstrate that they are doing their job honestly if they keep secret the names of those on whom chance falls? □



Britain leads bid to cut costs of Europe's space science projects

Paris. Having successfully helped to engineer a major reduction in planned expenditure by the European Particle Physics Laboratory on its Large Hadron Collider (see opposite), Britain is now on a collision course with the European Space Agency (ESA) over the future funding of European space science missions.

At stake is the level of support for the various projects included under ESA's Horizon 2000 programme, a series of missions that started at the beginning of the decade and are already being planned into the first years of the next century.

Last week, Roger Bonnet, the head of ESA's science programme, warned that ESA may have to postpone or cancel some of these missions if the United Kingdom insists on a reduction in the programme's overall costs. He was speaking after ESA's council had failed to agree on proposed future funding levels for the programme.

British officials say their main concern is to increase the cost-effectiveness with which the £37 million (US\$58 million) Britain pays each year towards ESA's space science programmes is used.

But Bonnet, who is not prone to making rash public statements, claims that Britain wants a "20 to 50 per cent" cut in funding of space science over the long term, and that this will inevitably lead to programme cuts.

According to Bonnet, the dispute takes place against a background of widespread

political apathy towards space activities among European countries. As a result, he warns that ESA is "in crisis", and that, in the absence of renewed political commitment, the long-term future of the agency itself is under threat.

Indeed, Bonnet suggests that the agenda of an interministerial meeting in 1995 should be enlarged into a full-blown European space conference to review the whole of space policy in the light of economic and political changes. "We need a new political commitment to space that is now lacking," says Bonnet.

One factor in the success of Horizon 2000 is the funding stability it enjoys. Whereas ESA's 14 member states can choose whether to take part in the agency's technology programmes, such as the development of the Ariane launcher, they are obliged to contribute a fixed proportion of their gross national product to the space science programme, and future funding levels are agreed for five-year periods.

But simmering tensions, triggered partly by budget pressures in the United Kingdom, boiled over at a meeting in Paris last week of

the ESA council, which is made up of representatives of each of the agency's member states. The meeting failed to vote on the level of funding for the period 1996–2000 after it emerged that the United Kingdom, Germany and Spain would oppose the figures proposed by the agency, based on increases at the rate of inflation. (Under ESA procedures, approval must be unanimous.)

The three countries had voted earlier in the meeting against the 1995 budget, arguing that this should be held to constant money, rather than growing at the anticipated rate of inflation. But the budget increase was still adopted, as it had already been agreed in principle under the current five-year plan, and therefore needed only majority approval. (Italy abstained from the vote, pending a review of its domestic national budget; see page 719.)

British officials have been quick to reject Bonnet's claims. "We are not seeking a reduced science programme," says Ian Corbett, director of science at the UK Particle Physics and Astronomy Research Council (PPARC), and the British representative on the ESA council.

But Corbett argues that ESA could cut 25 per cent of the costs of its science missions within five years, without reducing their scientific value, by making them more cost effective. He admits that Bonnet has a well-managed programme; "we're giving him 9 out of 10", says Corbett. "But saying that, you still have a bit to go."

PPARC's current difficulties stem from the fact that its budget for space science is being held level, while its subscription to ESA is increasing, leaving it with "less and less money" to spend on instruments or on exploiting the results of missions.

Bonnet says that he appreciates PPARC's problems, and is willing to seek ways to cut costs further, for example through the way contracts are shared among companies in the member states. Indeed an audit of the science programme, and ESA's general running costs, is already being carried out.

But he refuses to make cuts that could jeopardize the overall space science programme, and fears that Britain is looking on savings "as simply a means to reduce costs", rather than "as a way to do more science for the same money".

The question of how much funding Horizon 2000 will receive in its next five-year funding period will be considered by the space ministers of ESA member states at a meeting next autumn, which is also scheduled to decide on Europe's contribution to the planned international space station.

Declan Butler

S. Vennart/ESA

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**Bonnet: seeking to
defend programme.**

Australia hails a prehistoric pine

Sydney. Not the world's smallest Christmas tree, but — for those still seeking unusual Christmas presents — perhaps one of its first prehistoric pot plants. A research officer at the Mount Annan Botanic Gardens in Sydney, Australia, holds the first seedling (right) raised from a new species of pine, thought to have been extinct for 150 million years, which was discovered in a national park 125 miles west of the city in August.

The discovery was hailed in Australia last week as one of the most important finds of the century, equivalent, according to Carrick Chambers, the director of the gardens, to "finding a small dinosaur still alive on Earth".

The pine was discovered by a project officer with Australia's National Parks and Wildlife Service when he absailed into a 1,800-foot deep gorge during a weekend bush walk. In all, 42 trees were discovered at a location that is not being revealed. (see also page 719)

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Relief and euphoria greet decision on LHC

Munich. Particle physicists can celebrate Christmas secure in the knowledge that 20 years of high-level science lie before them. Last week, the council of the European Laboratory for Particle Physics (CERN) approved construction of the Large Hadron Collider (LHC), one of the largest basic science research projects ever undertaken.

The decision was greeted with a mixture of relief and euphoria by CERN staff, whose confidence that the LHC would be built has been deeply shaken over the past six months. As one compromise solution after another was rejected, it seemed that tough conditions demanded by Germany and Britain would never be met. Now, according to an agreement that meets almost all of the German/UK demands, the accelerator will be built in two phases according to a baseline programme that assumes no financial contribution from non-member states.

After the demise last autumn of the Superconducting Super Collider, a competitor to the LHC planned by the United States (see *Nature* **365**, 773; 1993), particle physicists have been pinning their hopes on Europe's proposed LHC.

Last June, CERN's 19 member states agreed in principle to build the accelerator, at a total cost of SFr2.6 billion (US\$1.98 billion), inside an existing tunnel that runs in a 27-km circle beneath the French-Swiss border at Geneva.

The financial package offered by CERN to its member states at that time was SFr500 million more than they would have been able to pay out of their annual contributions. Christopher Llewellyn Smith, CERN's director-general, wanted the shortfall to be made up by contributions from non-member states.

But Germany and the United Kingdom made a series of proposals to reduce costs and ensure that the project could be independent from outside contributions. First, they argued, the cost of the LHC should be pegged to 1995 levels to ensure it remained affordable, and the host countries, Switzerland and France, should pay ten per cent of the total costs — SFr260 million over ten years — in addition to their annual contributions.

The new agreement is based on zero growth in the budget between 1995 and 1997. Thereafter, assuming a 2 per cent inflation rate, member states will be asked to

pay only one per cent more a year until 2008. France and Switzerland, however, will increase their subscriptions by two per cent a year over this 10-year period, adding an estimated SFr123 million.

Taken on top of their promised host state contribution of SFr121.5 million (SFr57 million from Switzerland, SFr64.5 million from France), this means that they will indeed foot nearly 10 per cent of the total bill on top of their general subscription.

Germany and the United Kingdom also wanted voting rules to be changed so that a request from CERN to raise indexation could be overruled by member countries with a combined contribution to the CERN budget of one-third. (The two countries contribute around 22.5 per cent and 14 per cent respectively to CERN's budget.)

Third, Germany and the United Kingdom had asked for harsh reductions in the general CERN budget. The laboratory has now agreed to lose 600 of its 2,900 staff by the year 2005, saving an estimated SFr25 million a year. Combined with other previously agreed savings, this means that a total of SFr800 million will be trimmed from the general CERN budget by 2008.

The severity of the German/UK demands caused tension between member states. Some felt that Germany, the highest contributor to CERN, was using its financial muscle to hold the project to ransom. Many of the issues, says Filippo Menzinger, a spokesman for the Italian delegation, could have been dealt with earlier, without tying them to the LHC decision.

In addition to the budget-trimming exercises, the timetable for LHC construction and experiments has been adjusted to spread the costs over a longer period. The collider will now be built in two phases. In the initial phase, up to 2008, the machine will be constructed to run at an energy level of 9.3 TeV, by omitting one-third of the accelerator's planned 1,200 magnets.

Lynn Evans, the director of the LHC, says that this energy is still sufficiently high to answer fundamental questions such as why the Universe is constructed entirely of matter, rather than the predicted mixture of matter and antimatter, and to carry out research on the top quark.

After 2008, the remaining magnets will be installed to allow the LHC to achieve its top energy of 14 TeV. Particle physicists will finally be able to seek their holy grail, the Higg's boson, a particle whose purported existence underpins theories of mass.

These plans will be reviewed in 1997. If CERN receives firm offers before the review from countries that have already expressed serious interest — the United States, Japan, Canada, the Russian Federation, India and Israel — it will be possible to build the 14-TeV accelerator by 2005 as originally planned.

"I would estimate the chances of [an accelerated timescale] to be strong," says Llewellyn Smith, who is flying to Washington next month to begin formal negotiations with the United States.

Not surprisingly, officials in both Germany and the United Kingdom have expressed satisfaction with the outcome. The German minister for research, Jürgen Rüttgers, says that the decision gives particle physics a secure future, and with a considerable saving to the budget.

Britain's Office of Science and Technology, which coordinated the UK's role in the final negotiations, says that the savings there will be used to support domestic research.

Allison Abbott

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**Llewellyn Smith: to
adopt two-phase plan.**

Japan and US explore collaboration

Tokyo. It will be at least a year before it is clear whether Japan is in a position to contribute to the construction costs of the Large Hadron Collider (LHC), following Europe's failure to reach agreement on the LHC in June (see *Nature* **370**, 165; 1994).

In late 1993, Japanese physicists decided to back the LHC when the United States cancelled the Superconducting Super Collider (SSC). Earlier this year they were pushing with the Ministry of Education, Science, and Culture to win a budget for the LHC in the fiscal year 1995 (which begins in April). But the opportunity has now passed.

Hirohata Sugawara, director general of Japan's National Laboratory for High Energy Physics (KEK), now says that he will do his "very best" to emphasize the importance of the LHC and of Japan contributing to the construction of detectors and "even

to construction of the collider itself".

But it will be late August next year before a budget request can be made (for fiscal year 1996) and a decision by the Ministry of Finance would not be made until the end of December.

Meanwhile Martha Krebs, director of the office of energy research (OER) at the US Department of Energy, said last week that she was "very pleased" that CERN has made the decision to go ahead with LHC.

Krebs said that the United States was now taking two steps over its possible participation. First, an interagency group will meet shortly after the new year to develop the US approach to discussions with the European organization. Second, John O'Fallon, head of the OER's high-energy physics division, had already invited the CERN team to visit the United States for talks. **David Swinbanks & Colin Macilwain**

Cautious welcome for new head of Euro-patent office

Munich. After almost a year of political wrangling, the council of the European Patent Office (EPO) last week elected Ingo Kober, state secretary of the German Ministry of Justice, as its next president. But the choice of a German has sparked concern among the EPO's 3,700 staff, half of whom are based at the Munich headquarters, next door to the German patent office, one of the strongest national patent offices in Europe.

The two offices have frequently been in dispute over the better conditions of employment at the European office, as well as the possible decentralization of tasks, such as patent searches, from the EPO to national offices. The EPO staff union, which represents at least three-quarters of employees, has long argued that the election of a German president could increase internal conflict (see *Nature* 371, 371; 1994).

But such concern may be misplaced. Kober's election was unanimous, and he is a popular choice outside the EPO. EPO's 17 member states have had difficulty in replacing the current president, Paul Braendli — whose second consecutive five-year term expires in April — and have voted inconclusively on the issue several times over the past nine months.

One reason the vote was split was that Braendli, who is Swiss, had put himself forward for a further three-year period. To leave more time for negotiation, Braendli's term was extended in September by eight months to the end of 1995. Member states also had to acknowledge a higher political agenda and consider the geographical share-out of other key European positions. Germany, for example, recently lost its position as head of the North Atlantic Treaty Organization (NATO).

Many had expected the bargaining to extend well into next year. But after Switzerland withdrew Braendli's candidacy, Kober, who is 52, was last week voted in and will start his five-year term in January 1996.

Kober, who is a lawyer with a strong background in patent affairs, headed the German delegation to EPO from 1987 to 1991. Since 1991 he has been state secretary of the justice ministry, where he has worked for nearly 20 years, with particular interest in the legal aspects of patent protection.

His reputation as hardworking and competent, with a good ability to get along well

with people, will be crucial in addressing the concern of the EPO staff, who are frequently criticized in the German press for enjoying high salaries and special privileges, such as exemption from income tax.

These criticisms have been led by the Bavarian branch of the Free Democrat party (FDP), the small but influential political party supported mainly by small businesses and the self-employed, whose popularity fell in the October elections.

The president of the German Patent Office, Erich Häusser, is also an FDP member, and is known to be unhappy that employees in his organization are paid less than their EPO neighbours for doing similar work. The fact that Kober himself is an FDP member is not reassuring to EPO staff, who feel that Germany has never publicly defended their interests.

"We now want the German delegation [to the EPO] to stand up for us in public, and point out the economic benefits for Germany in having the EPO here," says Anders Sunnhagen, chairman of the union. If it does not, he says, staff will ask the next council meeting in June to debate a motion that Germany is not taking seriously its responsibilities as host country, and that all or part of the EPO should be moved elsewhere.

Kober says he will discuss the matter with the unions to try to defuse the situation. He claims that he fully understands the unions' view that their conditions of employment should be compared to other international organizations and not to the German patent office.

Kober says that as president of the EPO, his loyalty to the office will not be affected by national concerns. His main aim, is to improve the efficiency of the office, and to reduce the time involved in issuing patents.

He expects to continue the major changes introduced by Braendli, such as automating the office at a reasonable price — a sometimes controversial issue because of the high costs involved — and studying ways to reduce or restructure EPO's high fees, which small businesses and academics often cannot afford.

On the issue of decentralization, Kober believes that there is room for the limited devolution of tasks to national offices under existing EPO rules, but says he has no specific plans in mind.

Von Benthem confirms Kober's European credentials: "When he was a member of the German delegation to the EPO, he often went against the interests of the German patent office in favour of the EPO," he says. There is no reason to suspect that this will change when Kober becomes president, he adds.

Toni Feder & Alison Abbott

Promega files court challenge to Roche's *Taq* enzyme patent

London. Promega, the US laboratory supply company, has stepped up its fight against the patent owned by the Swiss pharmaceutical company Hoffman-La Roche on the thermostable enzyme *Taq* DNA polymerase by challenging the validity of the US patent in an action filed last week in a San Francisco court.

Promega claims that *Taq* polymerase, which is used in almost all molecular biology laboratories, was known to scientists before its 'discovery' by scientists at Cetus Corporation in 1985. In support of its case, the company cites in particular a thesis, written by a research student at the University of Cincinnati in 1974, describing the properties of a polymerase which it claims to be identical to that later purified by Cetus.

As other examples of 'prior art', Promega refers to work published in the *Journal of Bacteriology* in 1976 by a group of Cincinnati scientists, as well as the results of research by a group of Russian scientists published in the Soviet Union in 1980.

Similar arguments have already been rejected by both the US Patent Office and the European Patent Office (EPO) in Munich. EPO recently told Hoffmann-La Roche — which purchased the rights to both *Taq* and its most common application, the polymerase chain reaction (PCR) process, from Cetus for \$300 million in 1991 — that it intends to issue the company with a broad European patent covering thermostable polymerases (see *Nature* 372, 212; 1994).

Roche is also suing Promega for breaking a licensing agreement signed with Cetus. An attempt by Promega to head off this suit by challenging the validity of the patent has already been rejected by the courts. Many are interpreting the latest challenge as a new attempt by Promega to avoid paying the heavy costs of a licence violation.

But William Linton, president and chief executive officer of Promega, insists that a point of principle is at stake. "We believe that scientists [in the United States] should not be forced to pay a premium simply because a patent was granted based on erroneous and misleading information," he said last week.

According to Linton, "representations to the patent office of differences between the patented and the prior art polymerases made during prosecution of the [US] patent application are false".

But Agnieszka Junosza-Jankowski, licensing manager of Roche Diagnostic Systems, says the company considers Promega's arguments, which have already been addressed by patent officials, are "without merit". She adds: "We are confident that our position will prevail in court." **David Dickson**



Kober: denies any conflict in loyalties.

Geological programmes come under threat . . .

Washington. Earth scientists in the United States are growing increasingly concerned that the US Geological Survey (USGS) — the largest source of federal funding for US hydrologists, geologists and geophysicists — may be abolished in the new year unless they can persuade Congress that its \$600-million annual costs provide taxpayers with value for money.

The Republican threat to abolish the agency arose in a drastic budget-cutting

amendment in 1993. It reappeared in a minority budget proposal in March, and was then attached to the *Contract with America*, the platform on which the party won November's elections.

The survey is best known for mapping and geology, including assessing the risks from earthquakes and volcanoes, but its largest function is based on its responsibility to collect and assess hydrological data to help manage the US water supply.

. . . as Walker picks out new targets

Washington. The new Republican chairman of the House of Representatives Science Committee says that he plans to look closely at large-scale environmental research programmes, such as the National Aeronautics and Space Administration's (NASA)'s Mission to Planet Earth and other projects related to global warming, which he claims may be "more in tune with political priorities" than with good science.

Bob Walker (Republican, Pennsylvania) has also promised to encourage the National Science Foundation (NSF) to put greater emphasis on basic science, and to pursue legislation to enhance the role of risk assessment in environmental legislation.

Addressing a meeting of journalists and lobbyists in Washington last week, Walker was broadly supportive of existing government-backed research programmes, and short on proposals for drastic budget cuts.

But he said that the Clinton administration's fastest-growing research programme, the Advanced Technology Program, run by the National Institute of Standards and Technology, should "ultimately" be phased out. He also expressed concern that the costs of the proposed Tokamak Physics Experiment at the Princeton Plasma Physics Laboratory in New Jersey "have gone up 50 per cent", and suggested that fusion experiments were best carried out internationally.

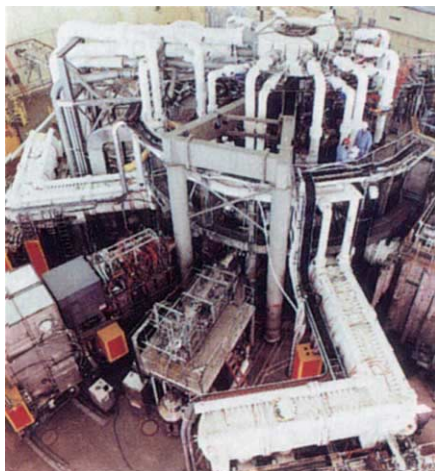
Walker has successfully fended off plans to rename his committee, formerly the Science, Space and Technology Committee, chaired by George Brown (Democrat, California), as the Technology and Competitiveness Committee. Instead, it will be known simply as the Science Committee.

Walker's close ties with the Republican leaders in the House, and his close personal friendship with the speaker Newt Gingrich (Republican, Georgia), in particular, should strengthen his committee's influence and help to improve its poor legislative track record.

In addition, changes in committee structure in the Senate, giving a greater voice to authorization committees, will make it easier than before to reach agreement on authorizing legislation. Even Brown concedes this

point. "Previously the Senate had shafted us," he says. "We now have people on both sides who want authorizing bills, and we will get them."

Walker pledged to continue Brown's campaign against congressional earmarking of funds for academic projects, and to hold early hearings at which agency chiefs such as Dan Goldin, the administrator of NASA, will be asked to describe their visions for the next century. He also said that the NSF, which has recently been under



Concerns raised over the increased costs of fusion research at Princeton (above).

pressure from a Democratic Congress to boost its education and applied research programmes, should be "far more concentrated on basic science".

But Walker's first priority is likely to be to promote legislation in the arcane but important sphere of risk assessment. He believes that new legislation is needed to devise a framework for the federal government's response to perceived environmental threats such as global warming, deforestation and endangered species.

Such legislation, although highly technical, will be extremely contentious. It will attempt to lay a basis for action in such fields in terms of demonstrated cost-effectiveness, based on proven science, rather than on political commitment. **C. M.**

The few congressmen who have so far voiced objections to the proposed abolition are Californians concerned about the future of the survey's work on the likelihood and impact of earthquakes, said a spokesman for John Kasich (Republican, Ohio), the chairman of the House of Representatives Budget Committee. It was committee staff members who put the survey on the hit-list.

But the spokesman emphasized that the list was intended only to exemplify what sorts of budget cuts could be made. "We did not say 'you have to use these cuts'," he said. "If people have other suggestions, we are ready to hear them."

The American Geological Institute (AGI), which represents 80,000 Earth scientists, is planning a campaign to defend the survey, as is the American Geophysical Union. Craig Schiffries, government affairs director at AGI, warns geologists to be aware of the dangers of "a pyrrhic victory, where [the Republicans] don't abolish the agency but cut its budget by 30 per cent".

Gordon Eaton, director of the survey, says that its failure to explain its functions adequately to the public is part of the present problem. "All of them relate to public health, safety, and the economic well-being of the nation."

Eaton has won assurances from his boss, Bruce Babbitt, the Secretary of the Interior, that the agency has the full support of the administration. But the threat to the agency is partly motivated by Republican loathing of Babbitt — the official in the administration most closely associated with environmentalist thinking — and he may not be well positioned to provide protection.

Two other science agencies in Babbitt's department, the National Biological Survey (NBS) and the Bureau of Mines, are also threatened with closure. The USGS will spend \$362 million on research and development this year, the NBS \$167 million, and the Bureau of Mines \$101 million. Most observers feel that the two smaller agencies are more vulnerable than the USGS.

As a heavily staffed federal agency with offices and collaborations in every state in the union, the survey may seem the typical sort of agency that Republicans would like to close. But unlike many federal agencies, it has a strong record of performing high-quality intramural research.

The survey has established centres of research excellence in the Earth sciences whose publication rates are as high as university centres, says George Brown (Democrat, California), now ranking minority member of the House of Representatives Science Committee. "That's the sort of thing we ought to be encouraging," he says — adding that he does not believe that there is "a chance in a million" that USGS will actually be abolished. **Colin Macilwain**

Call for risk/benefit study of gene therapy

Paris. The group that advises the European Commission on ethical questions related to biotechnology has called on the commission to make basic and clinical research in gene therapy a "priority". Its recommendation is part of a opinion on gene therapy made public last week in the presence of Jacques Delors, the outgoing commission president at a press conference in Brussels.

But the group also says that more attention should be paid to evaluating the risks and efficacy of the technology. The current publicity about somatic gene therapy trials risks "misleading the public", says Noelle Lenoir, the chairwoman of the group and a member of the French constitutional council.

"What we are saying is that gene therapy is a promising technology, so let's go for it," says Lenoir. "But let's remember it's still an experimental technology, so don't give false hopes, and be prudent at the research and clinical level." A full review of clinical trials already under way should be made

public, she says, in order to provide a "realistic assessment" of the risks and state of the technology currently available.

Lenoir argues that the tendency of some researchers to encourage "excessive" publicity about gene therapy has also been responsible for overreaction — both for and against gene therapy — among decision-makers, and risks altering the "prudence and rigour" of the scientific approach.

She also claims that trials are often "more medical than scientific", and often depend on small amounts of preclinical data, in particular when trials involve terminally ill patients. This narrow approach leads to neglect of the wider social issues raised concerning the social implications of individuals whose genes have been modified.

To correct such trends, the group recommends that national supervisory bodies make public regular assessments of continuing trials. It also calls for standardized European guidelines for good practice in gene therapy, with the European Medicines

Agency as an appropriate focus, and recommends that Europe-wide evaluations of the risks and results of gene therapy technology should be published regularly.

The group is also concerned that existing European Union (EU) legislation on the safety of genetically modified organisms (GMOs) is inadequate with respect to gene therapy. In particular, it claims that the wording of the two directives on the contained use and environmental release of such organisms means that the directives are not applicable to gene therapy. The commission should amend them to include a specific reference to gene therapy, says Lenoir.

Because of the uncertainty of the risks of somatic gene therapy, the group also recommends that it be restricted to serious diseases for which no other treatment is available. Widening to other "therapeutic" applications, it says, should be considered only after a case-by-case medical and ethical analysis, it says.

The group says that human germline gene therapy "is not ethically acceptable at the present time" given the "important controversial and unprecedented" questions it raises.

This compromise formula — which accommodates both those who seek a moratorium on such techniques and those who consider a ban premature and unnecessary — reflects a growing belief that this debate serves little purpose other than to detract attention from the more immediate and tangible problems of somatic gene therapy.

The opinion also recommends that Europe should establish the equivalent of the US Orphan Drugs Act, which provides commercial and legal incentives to companies that develop drugs for rare diseases. This reflects concern that gene therapy will eventually be used mainly for common diseases such as cancer and AIDS, and that pharmaceutical companies will abandon research into rarer diseases because it is no longer profitable.

But Lenoir says the main aim of this recommendation is formally to introduce the notion of equal access to therapies as an ethical issue. She points out that the group has the right to demand this from the commission, given that article 129 of the Maastricht treaty gives the commission a mandate to guarantee fair allocation of health-care resources.

The group is trying to shake off the accusation that it was set up by the commission only to confer legitimacy on the commission's often controversial biotechnology policies. Lenoir admits this perception exists, and is keen that the group should build a reputation as an independent watchdog.

Declan Butler

US to cut applied energy research

Washington. The US Department of Energy (DoE) is to bear almost half of the \$24 billion, five-year budget cutting package proposed by President Bill Clinton on Monday. \$1.2 billion of the savings will come from the department's applied research programme.

The cuts in applied research will be spread across a range of programmes, including nuclear fusion, nuclear fission, solar power and energy conservation. Department officials said that the \$40 million-a-year clean coal programme will be closed as ongoing projects in it are completed.

Other programme closures have not been specified. The department has merely said that the money will be found from cuts in its applied research programmes, and through cost-sharing and cuts in "lower priority programmes."

Bill White, the deputy energy secretary, said that decisions over whether to close any of the DoE's national laboratories would await the verdict of a panel chaired by Bob Galvin, former chairman of Motorola, which will report on the future of the laboratories in February.

A more detailed picture of the cuts will emerge on the return of Hazel O'Leary, the energy secretary, from a trip to Europe. But full details will not be released until Clinton publishes his budget proposals in early February. Some observers predict that few cuts will be specified even then, as most of these are likely to fall toward the end of the five year period.

The \$1.2 billion to be cut from DoE research will come from energy supply,

fossil energy and energy conservation work, department officials said. Their largest and most vulnerable elements include solar energy (\$251 million this year), fossil energy research (\$352 million) and magnetic fusion (\$363 million). DoE basic research, such as high energy physics and nuclear physics programmes, are not affected by the proposed cut.

Charles Curtis, the assistant energy secretary, last week issued a flat denial of reports that the Princeton Plasma Physics Laboratory (PPPL) in New Jersey, which performs much of the fusion programme, was to close. Rumours persist that at least one DoE lab will shut. But White insisted that "we haven't identified any particular facility to be closed."

The largest cuts will fall on the DoE's \$7 billion-a-year programme to clean up its nuclear sites. White said savings would come from subcontracting work to the private sector, although most is already done by contractors, and observers expect the cuts to further slow down the already glacial clean-up process.

Clinton's \$24 billion cuts package will pay for one-third of the middle-class tax cut which he promised the American people in a televised broadcast last week: the other two-thirds are to be paid for by extending an existing budget freeze out from 1999 to 2001. The package is seen as the starting point in a bidding war between the administration and the new Republican majority in Congress, in which the Republicans are likely winners and the science budget is a certain loser.

Colin Macilwain

Soros foundation offers its help free to other agencies

Moscow & London. The International Science Foundation (ISF), the body set up by the financier George Soros to support scientists in Russia, is claiming that "logistical bottlenecks" will cause significant delays in the payment of up to US\$30 million of similar aid that other Western agencies have offered to provide next year.

To prevent this happening, ISF is offering such agencies the free use of its own system for transferring research funds and scientific equipment to Russian scientists. Soros has personally agreed to underwrite the costs involved.

But the offer has been coolly received by INTAS, the main agency concerned. Officials at INTAS, which was set up by members the European Union and six other European states, are prepared to sanction transfer of aid to Russia "by any legal means". But they are concerned that the tax exemptions secured by ISF for its grants may not prove legally valid for INTAS grants. (Under Russian law, salaries alone are subject to tax deductions of up to 53 per cent).

Soros set up ISF in 1992 with \$100 million, and the agency has since developed a large network of Russian and Western scientists. Its Moscow office employs about 70 staff, and an office in New York monitors all grants and arranges for these to be transferred to Russian scientists in hard currency.

But Soros's own money is due to dry up at the end of next year, and ISF is keen to maintain the infrastructure it has built up. Several months ago it set up a Grant Assistance Programme to offer other organizations the use of this infrastructure, including the tax-free importation of scientific equipment and materials, at a flat 10 per cent handling fee (see *Nature* 370, 242; 1994).

INTAS, which has so far rejected ISF's offer, remains equally sceptical of the new invitation. "We cannot guarantee that money transferred through ISF will not later become subject to tax," says Pierre Venet, director general of INTAS. INTAS admits it is still refining its own procedures for transferring funds, and that these have yet to be officially approved by the Russian authorities. Venet stresses that INTAS's activities must have protection under Russian law, as it is an intergovernmental organization.

Venet adds that once INTAS procedures are running smoothly and have obtained full legal protection, it may require these to be used by all its grant recipients, rather than alternatives such as ISF's system. One reason, he says, is that a centralized procedure will allow INTAS to keep full records of how its grants are being used.

But ISF officials claim that INTAS's concerns about the legality of the founda-

tion's procedures are misplaced, and that the reluctance of the European body to take up its offer is political — reluctance to see European aid funds handled by a US body.

Meanwhile, Russian authorities remain divided about the activities of the ISF, even though the government has offered \$12.5 million to the foundation, to be matched by a similar amount from Soros. One explanation is that some Russian officials say they would prefer to rely on mutually beneficial cooperation agreements in science rather than on Western charity.

Irritation about ISF's activities — which ISF officials attribute to factors ranging from the foundation's low overhead costs to its tax-free status — emerged at a meeting in



Brussels in October on the obstacles to cooperation in science and technology with the Russian Federation.

Gennady Mesyats, chairman of the Urals Scientific Centre of the Russian Academy of Sciences, for example, told the meeting that the West should thank Russia for giving it access to technology. He claimed ISF had been created by *émigrés* who disliked the Russian Academy of Sciences, and wanted to prevent it from having a role in distributing funds. The new grant assistance programme should be ignored, he said, and funds transferred directly to the institutes employing grant recipients.

Nevertheless, ISF officials claim that many Russian scientists who were initially sceptical about the foundation now support its activities. Alex Goldfarb, the executive director of the ISF, claims that such support was evident from a number of leading officials of the Russian Academy of Science at a meeting of the academy in Moscow earlier this month. "They now accept that they were wrong in their initial assessment of what we are doing," he says.

Vladimir Pokrovsky & David Dickson

US and Russia agree joint projects, but not funding agency

Washington. US and Russian negotiators have signed 15 new agreements on cooperative projects in areas including science, space, and environmental monitoring. But at a meeting in Moscow last week, the US team, led by Vice President Al Gore, said it was unable to support a joint research and development foundation — despite earlier White House hopes that a domestic dispute over US funding could be solved in time for the meeting (see *Nature* 372, 584; 1994).

The newly signed agreements include a plan to collaborate on the prevention of pollution in the Arctic, to fly two US stratospheric monitoring instruments on Russian satellites, and to cooperate on the Acoustic Thermometry of Ocean Climate (ATOC) experiment run by the US Advanced Research Projects Agency.

A new US-Russia Health Committee announced joint programmes to support partnerships between US and Russian pharmaceutical firms and to foster women's reproductive health. The committee also agreed on priorities for cooperation, including diabetes, health education, control of infectious diseases, and primary care practice.

According to Jeff Schweitzer of the White House Office of Science and Technology Policy, the joint R&D foundation is "not completely dead". But it appears unlikely to gain approval. Lawyers for the Department of Defense (DOD), which was to have paid \$10 million into the fund this year, continue to insist that the law creating the proposed foundation requires another federal agency to come up with matching funds.

The DOD also is holding back on continued US funding of the International Science and Technology Center (ISTC) in Moscow, a competitive grant programme funded jointly with Europe and Japan in order to provide former Russian weapons researchers with more peaceful work.

The centre has committed \$49 million this year — about half of it from the United States — to support more than 5,000 individuals working on 94 different projects. But that money is nearly all used up, and no US funds have been allocated for next year.

Anne Harrington of the State Department, which is responsible for American involvement in the ISTC, says merely that the matter is "under discussion" at several agencies — including DOD, which paid most of this year's contribution through "reprogrammed" defence funds.

Tony Reichhardt

UK scrutiny of public labs under fire from Parliament

London. A select committee of the UK House of Lords is the latest body to raise strong objections to both the procedures and conclusions of a civil service inquiry into the 'efficiency' of a number of publicly funded research laboratories.

The committee's criticism could prove the final nail in the coffin of the government's apparent enthusiasm for the privatization of a significant number of these laboratories (including their possible 'purchase' by universities). Reforms, though still anticipated, are likely to be evolutionary rather than revolutionary.

The so-called 'efficiency scrutiny' was carried out earlier this year by a group of civil servants. The Cabinet Office's efficiency unit asked them to look at 53 government laboratories, and in particular to explore which of them might be privatized.

Although its preliminary report, published in April, found little scope for widespread privatization, the scrutiny team did suggest two ways of grouping the laboratories — either by geographic region or by scientific discipline — as well as various other changes (see *Nature* 368, 681; 1994).

But in a report published last week based on evidence presented by scientific bodies and other organizations, the House of Lords Select Committee on Science and Technology criticizes virtually all of the review team's proposals as unnecessary and potentially damaging to the UK science base.

The only conclusion it supports is that there should be a greater flexibility in Treasury rules, allowing research establishments to operate more commercially. But it rejects both of the alternative models proposed by the review team for grouping the laborato-

ries, criticizes its "narrow terms of reference", and describes the choices of establishments scrutinized as "haphazard".

"We believe that the whole report was misconceived," says the Earl of Selborne, the chairman of the select committee, and himself a former chairman of the Natural Environment Research Council. "It was reasonable for the government's efficiency unit to scrutinize an area of major public expense; but the way this exercise was carried out was totally inappropriate."

Selborne said that the committee had been particularly concerned that the team's original terms of reference placed a stronger emphasis on privatization than on any other model of reorganization, adding that "the exercise appears to have been Treasury-led rather than science-led".

The report itself, echoing sentiments from bodies ranging from the Royal Society to the Institute of Professionals, Managers and Specialists (the labour union representing most of the staff in the bodies being scrutinized), continues: "We do not believe that sufficient attention has been paid to the question of the effectiveness of public sector science in the pursuit of wealth creation and quality of life, without which any study of the efficiency of the management of that science has little value."

Speaking last month in front of a House of Commons committee (which later published an equally critical report) David Hunt, the new cabinet minister for science, said that he had an open mind about his eventual reaction to the scrutiny team's recommendations. But there now seems little prospect that the major changes it proposed in April will be adopted full-scale. **David Dickson**

Research council to require plan of proposed projects

London. Britain's largest research funding agency, the Engineering and Physical Sciences Research Council (EPSRC), is to require all grant applicants to provide a diagram on a single sheet of paper demonstrating how they intend the goals of their research project to be achieved.

The project will subsequently be evaluated partly on the basis of its success in achieving these goals. The move represents part of a new strategy being developed by EPSRC officials to increase the effort that goes into assessing the output of research projects — not merely their scientific potential.

Some scientists, while welcoming the council's attempts to increase the cost-effectiveness of its grant-giving practices, are wary of possible dangers in placing excessive emphasis on the achievement of pre-selected goals. "I would be worried if researchers started to play safe with their applications, only stating goals that they were confident of achieving" says physicist John Mulvey of the group Save British Science.

But Alan Rudge, the chairman of EPSRC, told a meeting of industrialists in London last week that in the past, while a great deal of effort had been placed on selecting grants for funding, relatively little had been spent on auditing what had been achieved. "I think that we have had the wrong balance," he said.

According to EPSRC officials, all grant applicants will therefore in future be asked to identify the criteria by which they would like the outcome of their research to be judged. Included in this process will be a one-page diagram of "the logic of their proposal". If a scientist feels unable to produce such a chart, "then peer reviewers will have to say whether they agree with that assessment".

Rudge claims that, although the evaluation of basic research needs to be approached with a "slightly different attitude" to applied research directed at specific goals, "it is an illusion that you cannot have any peer review of the way in which you intend to go about a programme." Even basic researchers, said Rudge, needed a plan of the route they intended to follow. "When you audit a project, you audit it against a plan," he says. "Even basic research has an objective."

Another proposal being studied to increase the effective evaluation of EPSRC-sponsored research projects, said Rudge, is the organization of 'theme days' during which EPSRC-sponsored research groups would demonstrate their work to interested industrialists and other potential customers for their results.

Research body revives 'Faraday' concept

London. The UK Engineering and Physical Sciences Research Council (EPSRC) is reviving proposals to create a network of 'Faraday centres' — named after the nineteenth century British physicist, and modelled loosely on Germany's Fraunhofer Institutes — to act as a bridge between industry and academic institutions.

The proposals were first made in 1991, by a commission headed by the Prince of Wales, and revived by the Conservative government during the 1992 general election campaign. But Michael Heseltine, the president of the Board of Trade, later dropped the idea on the grounds that the government had little interest in new institutional initiatives.

Last week, Alan Rudge, the chairman of EPSRC and board member with re-

sponsibility for research and procurement at BT, told a meeting of industrialists that it was time to take another look at the idea. "We are studying it closely to see whether it could be encouraged jointly with the Department of Trade and Industry."

The attraction of the 'Faraday principle', he said, is that it would provide a way of building up 'intermediate institutions' with links to universities and other academic bodies on the one hand and with industry on the other.

At the same time, Rudge warned universities that placing excessive emphasis on intellectual property rights of discoveries risked impeding efforts to build closer ties with industry. This could "slow the whole process." □

Italian space scientists face more upheaval

Rome. The Italian Space Agency (ASI) could be shaken up for the second time in just over a year if Stefano Podestà, the minister for research and universities, has his way — and the Italian coalition government survives its current turmoil. Earlier this month, the senate committee for industrial affairs approved a bill, drawn up by Podestà, to install for one year an *amministratore unico* — a single administrator — with a brief to restructure the agency.

The move has precipitated a fierce battle in parliament over Podestà's motives, while scientists are concerned that uncertainty over the agency's short-term future could mean that the distribution of grants for space research once again grinds to a halt.

It is the second time Podestà has tried to change the way ASI is run. In November he tried to push a decree through cabinet to achieve this objective. But the move failed on the grounds that a decree, which avoids a parliamentary debate, can only be used in emergencies.

Podestà has subsequently tried to rush his bill through parliament. He successfully arranged for it to be approved by the senate industrial committee, with the result that it did not have to be discussed by the full senate. However, following pressure from parliamentarians who want to ensure that it gets a proper debate, the bill will now be discussed in detail by the chamber of deputies, rather than just by a committee.

Two hostile parliamentary questions have been put, one challenging the appropriateness of the proposed restructuring, the other asking Podestà whether his motive in rushing through this legislation was partly to allow him, in return for a previous favour, to appoint the current ASI director general, Mario Calamia, to the new post as the sole administrator.

ASI has been plagued with problems since it was set up in 1988. It has always been financially disorganized, and became

deeply embroiled in corruption scandals last year, while an internal dispute over the amount of money ASI is supposed to give to basic research had blocked funding for space research for nearly two years.

In August 1993, the government approved a decree to install a commissioner to set ASI's administrative and financial affairs in order, and money started to flow to space scientists. But the commissioner was unable to get ASI's books to balance.

The situation appeared to be more stable when the then research minister, Umberto Colombo, appointed Giorgio Fiocco, an atmospheric physicist from Rome University, as ASI president last March, a few days before the general elections. Fiocco belatedly appointed a new science committee in the summer, and this convened last month to finalize allocation of the 1994 budget, which has remained unapproved.

But Podestà is now said to want to replace Fiocco with his own candidate — rumoured to be Calamia, who was appointed by Podestà in early summer. He has never discussed ASI's affairs with Fiocco, even though he regularly meets the heads of Italy's other research organizations.

Podestà says that turning ASI into "a healthy body for space research" is a necessary part of the overall restructuring of Italy's research bodies (see *Nature* 372, 393; 1994). A major task of the new administrator will be to try to disentangle ASI's financial problems.

In the early 1990s, ASI took on more commitments than it could afford. As a result, either some existing contracts with Italian industry will have to be revised, or more money must be provided by the government to fulfil the agency's legal obligations. According to the bill before parliament, the single administrator will be helped in tackling this issue by three economists from the treasury.

But scientists are concerned about several aspects of the bill. In particular, they are worried that the new temporary structure being proposed for the administration of the agency would give an excessive amount of power to the research minister.

According to the bill, the single administrator will be appointed by the minister. The latter will also have the power to decide how much money is allocated to basic science (the current law stipulates that this should be 15 per cent of Italy's space budget), based on the advice of a committee of "three experts in research and two economists", each appointed by the minister. (A new science committee will be appointed directly by the administrator.)

In addition, Podestà plans to appoint a separate committee to investigate the activities of ASI between 1988 and May this year. This covers the period in which the former government's commissioner was in place, and will presumably work in parallel with a current court inquiry into ASI's affairs.

Alison Abbott

Italy drops decision to quit EMBL

Munich. The Italian government has formally announced that it is withdrawing its notice to leave the European Molecular Biology Laboratory (EMBL), based in Heidelberg, at the end of this year.

Italy's notice that it was leaving EMBL was based on its unhappiness over the relatively low number of Italian staff scientists at EMBL, despite the fact that it pays around 16 per cent of the laboratory's annual budget.

The decision to return to the fold, which has been expected for several weeks, is linked to plans to establish a joint European Union/EMBL mouse gene repository near Rome (see *Nature* 372, 305; 1994). **A. A.**

'Fossil tree' reveals full splendour

Sydney. **The full-grown version (right) of a prehistoric pine tree found in August in a secluded rain forest in the Wollemi National Park west of Sydney (see also page 712). According to scientists at the Royal Botanic Gardens in Sydney, the Wollemi pine is a newly discovered genus whose nearest relatives died out in the Jurassic and Cretaceous eras 195–140 million and 140–65 million years ago respectively.**

The trees, about 40 metres tall and with a girth of three metres, are covered in a dense, waxy foliage, and have "a distinct nobbly bark that makes them look like they are coated with bubbly brown chocolate".

Chris Hartcher, environment minister for New South Wales, has described the discovery as "the Australian Christmas tree". Scientists have compared the significance of the discovery to that of the coelacanth discovered off the coast of Madagascar in 1948. □

IMAGE
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REASONS

One step forward, two back?

SIR — Although the new European Science and Technology Assembly (ESTA) has been discussed in *Nature* (see 371, 190; 372, 389–390 & 395; 1994), one distinctive feature has not been mentioned. Among its 100 members there are more Nobel prizewinners (six) than female scientists (four). How is such a committee put together, and how in the 1990s can women be so poorly represented on this top level European Union (EU) committee, which has the task of “improving the quality and choice of European Union Research Programmes”, and which will have considerable influence on how EU research policy is developed and how EU research money is spent?

The 27 members of CODEST (Committee for the European Development of Science and Technology) automatically became members of ESTA. The other 73 slots were filled by asking the European organizations listed in the table to nominate individuals. Organizations were asked to nominate approximately twice the number of individuals as places, with the final selection of ESTA members being made in Brussels. This was to give the commission a chance to balance ESTA by country and by field. Not surprisingly, ESTA is dominated by the larger countries, with Germany supplying 16 members, France 14, the United Kingdom 11 and Italy 10, but even small countries such as Iceland and Luxembourg have one member each.

In this context it is instructive to look at how the four female scientists came to be included, and from which countries they come. Dervla Donnelly, a phytochemist from University College, Dublin, Ireland, is included as a member of CODEST. Consuelo de la Torre, a plant scientist from CSIC in Madrid, Spain, and Birgit

Grodal, an economist from the University of Copenhagen in Denmark, were nominated as 2 of 6 members proposed by the Academia Europaea, while Margarita Salas, a geneticist from CSIC in Madrid, Spain, was nominated as one of 13 ESTA members directly chosen by the commission. In contrast, all the other organizations listed in the table — including the European Science Foundation which supplied 12 members — either did not nominate a woman or the woman was not selected. Indeed, women are not only poorly represented by number on ESTA, but once again there are no women from the major EU countries, Germany, France, the United Kingdom and Italy.

In spite of numerous resolutions from the council, the commission and the European Parliament endorsing equal opportunities, little specific effort seems to have been made to find female scientists qualified to serve on ESTA. This can be seen from the list of organizations asked to nominate individuals, which come mostly from the physical sciences, and from industry (see table), where there are even fewer women at the top than in the life sciences. Second, given the current gender distribution in senior science jobs, unless a call for nominations is accompanied by a request to include a certain proportion of women among the nominees, women are unlikely to be nominated on sheer statistical grounds. In contrast, informal efforts last year to put together a list of female scientists from EU countries qualified to serve on top EU committees showed that there was no lack of qualified candidates. Indeed, after asking both male and female scientists to suggest names it was relatively easy to assemble a list of more than 50 women from 11 of the 12 EU countries.

Is 4 per cent a reasonable number? The commission would probably argue that it is, as national and European academies rarely have even this percentage of women members. Yet if one uses this as the only guideline it is a depressing outlook for half the human race, as such organizations perpetuate themselves and are therefore very slow to change. Instead, with ESTA an opportunity has been missed to implement the call in the 1993 EU Workshop on Women in Science, and elsewhere, for more representation of women on the top EU committees that set policy and control funds. If, for example, 10–15 per cent of ESTA members had been female (which would have meant identifying only 6–11 additional women with the necessary qualifications), a signal might have been given to other EU and national committees on the need for more gender inclusiveness. This would also have gone some way to meeting the call in the British government's committee report, *The Rising Tide*, for a 25 per cent representation of women “for all public appointments and senior positions in science, engineering and technology, including chairmanships” by the year 2000. And a little way towards the situation in the United States where, already in 1994, 6 out of 19, or 32 per cent, of the members of the recently announced President's Committee of Advisors on Science and Technology (PCAST) are female.

In summary, the ESTA composition illustrates once again the gulf that exists between words (which are cheap), and actions (which require foresight and commitment) when it comes to implementing equal opportunities.

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Members proposed by:

	No. of members	No. of women
CODEST	27	1
European Science Foundation (ESF)	12	0
European Industrial Research Managers' Association (EIRMA)	12	0
Academia Europaea	6	2
All European Academies (ALLEA)	6	0
European Rectors Conference (CRE)	6	0
European Council of Applied Science & Engineering (EURO-CASE)	4	0
Industrial Research & Development Advisory Committee (IRDAC)	4	0
UNICE	2	0
European Round Table (ERT)	2	0
European Trade Union Confederation (ETUC)	2	0
CERN	1	0
European Space Agency (ESA)	1	0
European Southern Observatory (ESO)	1	0
European Synchrotron Radiation Facility (ESRF)	1	0
Direct Appointment by European Commission	13	1
	100	4

The breakdown by country (in brackets the number of women) is Germany 16 (0), France 14 (0), UK 11 (0), Italy 10 (0), Spain 7 (2), Netherlands 7 (0), Belgium 7 (0), Ireland 5 (1), Denmark 4 (1), Sweden 4 (0), Switzerland 4 (0), Austria 3 (0), Finland 2 (0), Portugal 2 (0), Greece 1 (0), Norway 1 (0), Iceland 1 (0), Luxembourg 1 (0). ESTA members are listed by country where they are currently working rather than by nationality.

...still no silence?

SIR — Not being a native speaker of the non-phonetic language English, I appreciate the discussion of the correct pronunciation of certain scientific terms. Norby or Busser and Horowitz argue convincingly (*Nature* 372, 312; 1994) that its greek root and ‘pt’ at the beginning of a syllable determine silencing of the ‘p’ in ‘apoptosis’. However, as soon as I apply this rule to the scientific term for butterfly (as ‘feather-wings’) — ‘Lepido-ptera’ — my confusion is back; Webster suggests the pronunciation ‘lepidoptera’. Am I missing an exception to the rule?

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How to pursue academic excellence

The last of *Nature's* celebrations of its 125th anniversary, a conference in Japan last week, uncovered Japan's discontent with the condition of the national universities.

Tokyo. When one speaker at last week's *Nature* conference here described it in a hackneyed phrase, "the end of the beginning", to what process can he have been referring?

The flowering of basic research on the Pacific rim perhaps, as might have been suggested by the title "The modern university as a centre of excellence" and by the job descriptions of the participants, many of them technically inclined university officials from China, Hong Kong, South Korea, the other China across the China Sea from the mainland whose capital city is Taipei, not to mention Japan, Western Europe and the United States?

The reasonable guess is wrong. What the speaker meant was that endless talk of reforming the national universities of Japan may give way to action now that other countries in the region have shown that dynamic higher education systems can be built on the eastern seaboard of the world's largest ocean. But the speaker is an optimist. Others believe that Japan's universities will stay the way they are for a long time yet.

To anticipate what follows (which is a necessarily over-brief account of two days of discussion), the issue of Japan's universities was not an explicit part of the agenda, but became so at the end of two days of discussion, as Japanese participants could no longer bear the frustration of hearing of rapid change among their neighbours. The difficulties in Japan, which are well-known (see, for example, *Nature* 359, 573-582; 1992) are that the bureaucratic control of the Ministry of Education and Culture is oppressive, and that the legal autonomy of professors gives them the illusion of academic freedom at the expense of the coherence of the university as a whole.

Here is how the argument developed. Dr Donald Langenberg, Chancellor of the University of Maryland System (comprising 13 campuses of different character) explained why he believes that, for the research enterprise in the West, "the party's over". Whether present federal spending in the United States is a plateau or will decline remains to be seen (but the new Republican majorities in the US Congress are not good omens). But, whatever happens to research, universities will increasingly have to match the increasingly varied demands of their increasingly diverse customer-students (no longer mostly teenagers).

But will not US institutions, with the

strength they derive from diversity, be saved by the impending enlargement of the teens age group? Not really. Their demands, especially for an education they or their parents can afford, will compel all institutions to pay more attention to what and how they teach. The "sage on the stage" has had his day. Bill Gates and Microsoft Inc. may yet successfully subcontract for educational services, or even become a competitor. "Re-engineering the research university" is the immediate goal. "Change is the only constant." Except in Japan, he might have added.

But is not change the enemy of the academic freedom of individuals and the autonomy of institutions? Mathematician Sir Peter Swinnerton-Dyer, successively vice-chancellor of the University of Cambridge, chairman of the British University Grants Committee and founding "CEO" of its successor, the Universities Funding Council, defined those concepts in the modern context*.

Academics have the right (and even the responsibility) to express and to teach unpopular views if they believe them to be true, for which only freedom from the fear that they may be dismissed for so doing is a protection. (Swinnerton-Dyer inclines to the narrower definition that the freedom applies only within the fields in which an academic is expert.)

That implies tenure, at least for those for whom teaching is an integral part of academic work. In passing, Swinnerton-Dyer remarked that British universities, in which tenure was extinguished for new appointments by government initiative in the 1980s, continue to behave as if it were still a reality.

By extension, the argument continued, academic institutions must be free to decide what to teach, and how, as well as to appoint their own staff. They should also have, "within the limits of prudence", the right to decide how their income shall be spent; "not too large" a proportion of total income should be earmarked for specified purposes.

Pointedly, Dr Akito Arima, president of the University of Tokyo until two years ago and now director of Japan's unique free-standing research institute RIKEN, asked what can be done about academics who have ceased to function well. Swinnerton-Dyer's view is that tenure should never be given to people whose responsi-

*The full text of this talk will appear in a forthcoming issue.

bilities are exclusively for research, and that "you can put a fair amount of moral pressure on people (but some can be too senile to recognize it)". He also added that tenure should only be given after a trial period of several years.

In Japan, one of the notorious difficulties is that academics, employed as they are by the Ministry of Education and Culture ('Monbusho'), can easily shrug off pressure, moral or otherwise, because tenure (for life) is granted from the outset of a Japanese academic's career.

Professor Gisbert zu Putlitz, Rector of the University of Heidelberg from 1983 to 1987 (and now again a professor of physics there) argued the case for research within academic institutions and that for regarding universities as uniquely global institutions, capable of surviving social upheavals (wars included) when

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they have the freedom to plot their own course in the world.

Among his preconditions for excellence are the intellectual quality of students and staff, academic freedom ("including the right to make mistakes and to correct them"), freedom from bureaucracy (at best "a nuisance") and flexibility, both in being able to change direction as circumstances change and in being able to "change the structure". (Zu Putlitz cited the case of the microbiology institute at Heidelberg, created during his spell as rector, which is an integral part of the university, but where nobody has tenure.)

Dr Emilio Picasso, director of the Scuola Normale Superiore at Pisa (and the project leader for the construction of the LEP particle accelerator at CERN, Geneva, from 1980) echoed the case for a link between research and teaching and

The other China

Dr Yuan Lee is the Nobel prizewinning chemist from Berkeley, California, who has returned to the 'other' China, the one whose capital is Taipei, to be president of the Academy of Sciences there. He described it, last week, as a challenging task, implying that there is much to do before it becomes a centre of excellence.

Unlike most other organizations with this title, the academy is more a research institute in its own right than a sponsor of research institutes elsewhere. Lee says there are 1,500 academics on the staff, spending a total of US\$150 million a year and covering the humanities as well as science.

Assessment and evaluation is Lee's starting point, which he says has already uncovered the truth that the Chinese language department at Taipei has outstanding expertise and is likely to be further strengthened. He also plans to pay personal atten-

tion to the development of chemistry at Taipei, believing there is great potential in the field.

Last week, he spoke with feeling of the need for sympathetic management of young people at research institutions. "We are not very good at telling young people that they are good at what they do". Tolerance among colleagues is critical in the pursuit of excellence. In making appointments, institutions should consider "what will be good for the institution in five or six years time".

As conference organizers will readily appreciate, there are diplomatic problems to be solved when people from the two Chinas appear on the same programme. Last week, these were mostly solved because of the circumstance that Drs Lu (from Beijing) and Lee (from Taipei) became personally well-acquainted with each other when they were both living in the United States.

entranced his audience with colour slides (the leaning tower seen through a window in one of them) of the battered mediaeval buildings in which his unique institution is housed. It is a frankly élitist place, boasting of Volterra, Levi-Civita and Enrico Fermi among its alumnae, which was converted in Napoleonic times into one of the then-four *grandes écoles* of the Empire.

There is no single recipe for the education of an élite, Picasso modestly proclaimed, offering Pisa simply as one way of doing the job. His telling point was that Pisa's Normale is not a comprehensive university, but one skewed towards mathematics, physics, geography and the history of art by tradition and the scholarly interests of its present staff. In particular, 50,000 or so students are not a necessary condition of a university.

Something like that seems to be the ambition elsewhere, but especially on the mainland of Asia, where the numbers are unavoidably big and where people are busily creating universities overnight. In South Korea, according to KunMo Chung, president of the Institute of Advanced Engineering at Seoul, the whole university system has been reconstructed in the past three and a half decades, since the ending of the Korean War. An essential part of the recipe for the clutch of universities in Korea that have excelled is the recruitment of expatriate Koreans who have been educated elsewhere; Chung's own engineering PhD is from the University of Michigan.

Chung's message last week was of the globalization of the university, not merely because people are now mobile, but because of the Internet and the communications technology that has made that possible. Sooyung Chang, president of the

Pohang University of Science and Technology, explained that there are already 167 universities, but that South Korea still depends on the rest of the world (the United States, Britain and, in the future, Australia) for much of its graduate education. Engineering is the favourite area of study, and industry is a generous source of support for a few select institutions, such as Chang's, which is supported by a steel company, and Chung's, which is supported by the Daewoo group. But most South Korean universities are very poorly supported by the government.

Among many other things, KunMo Chung masterminds South Korea's programme to improve the status of the existing universities by means of a competitive programme for the creation of what are called — what else? — centres of excellence. "If you have centres of excellence already it's easy", he said last week. But in South Korea most research used to be second-rate.

The idea now is that groups of individuals (they must be drawn from at least five institutions) apply for a standard grant of US\$1 million a year for 9 years; a precondition for those in engineering is that they must have the promise of at least 5 times as much from industrial partners. The projects are mostly technically orientated (the industrial partners are looking for a return) and are externally reviewed every three years. Chung said last week that 35 of these centres have been created in just two years. One consequence, he said, is that South Korean academics have a new confidence.

The pattern of generous support for research in science and engineering within technically skewed institutions seems to be the general pattern in eastern Asia.

The flashiest development of all seems to be that of the Hong Kong University of Science and Technology (HKUST), a brand-new campus founded in 1988 on a finger of one of Hong Kong's islands dipping steeply into the sea, and now covered with some of post-modernism's most spectacular buildings at the other end of the architectural spectrum from those at Pisa.

Dr Chia-Wei Woo, the first (and present) vice-chancellor, is a man in a hurry. Last week, he explained that when he had taken the new job, he and his wife stayed up late every night building a network among other ethnic Chinese academics around the world, seeking to identify potential teachers. There are now 400 (90 per cent of them from the United States), but the number is growing at 10 a month.

HKUST is and plans to remain a single-minded institution. From the outset, it has had separately registered commercial companies to take charge of its industrial liaison, in which Hong Kong's industry is said to be an enthusiastic partner driven by the recognition that Hong Kong is in "economic transition from low to high-technology industry".

Woo reckons that each staff member already brings in \$50,000 a year by way of grants and contracts, for projects such as the transmission of television signals by telephone line and the provision of wind-warning systems for Hong Kong's airports. At the end of its first four-year teaching cycle earlier this year, the university awarded 576 first degrees, 212 masters' degrees and one PhD (a transfer from elsewhere). HKUST aims at 7,000 students within eight years, or to operate on the scale and in the style of Massachusetts Institute of Technology. Woo boasts that one member of staff has already been poached by another university.

China proper, inevitably, is a still bigger task. Yongxiang Lu, a vice-president of the Chinese Academy of Science, pointed out last week that China already has 1,075 universities and colleges (perhaps a third as many as the tertiary institutions in the United States), but that a third of the students study engineering, compared with a fifth in Japan and a twentieth in the United States.

But even the 1.29 million engineering students in China cannot meet the demands of the "socialist market economic system", so that growth is the order of the day. How to arrange for that while keeping the economy ticking over? Lu guesses that half of his own university's budget comes from industry. Universities willingly accept part-time appointments and encourage academics to set up technical companies (one of which has designed China's most powerful computer now being marketed by a Hong Kong company), and will take on development as well as research contracts.

In short, the new universities of east

Asia seem determined to be flexible, are determined to recruit the best people they can find as teachers and are conscious that much of their income in the long run will depend on the value that industry puts upon them. Of necessity, the university presidents who put their case last week are not the ones best qualified to speak of the freedom ordinary academics enjoy.

On assessment, participants last week offered a variety of opinions. For zu Putlitz, a prior consideration in assessing the quality of a group of academics is secular success: what is the age distribution, how often have people been offered jobs elsewhere (a plus), and how often does the group offer its own graduates permanent positions (a minus)? Only then does he think it worthwhile looking carefully at people's publications and the status of the freedom ordinary academics enjoy.

Minoru Oda, the founding director of Japan's institute of advanced study at Nara, near Kyoto, argued that the assessment of teaching is more difficult when the crucial function is "to convey a sense of excitement to young people", but Swinerton-Dyer (advocating assessment by more senior and more junior people as well as by students "who take the responsibility very seriously"), preferred to trust to instinct: "I know one [a good teacher] when I see one".

Langenberg, on the other hand, quoted with approval the decision of one of the three campuses in his system to hire a clinical psychologist to assist with the assessment of the teaching function. "Rarely do we use professionals", he declared. Chung, by contrast, held that a good teacher is one who "refrains from preventing the natural development of students" while Lee held that the best teachers were not necessarily those who explained things most clearly.

Some institutions in Japan are (according to Oda) preparing for the individual assessment of academics, but there is little

agreement on how it should be done. And nobody answered the speaker from Tsukuba who asked how teaching could flourish when the "rewards system" favoured research.

Where does that leave the universities of Japan, the first of the Pacific countries to make its economic mark? There are more than 100 public universities (most controlled by Monbusho, some by prefectures) and several hundred private universities (most of which earn a government subsidy, but whose students pay high tuition fees, and whose academics are often part-timers). There have been whispers of anxiety for several years, ever since Japan (still the richest country in the Pacific, perhaps the world) began looking over its shoulder at the industrial achievements of its neighbours.

Last week, discontent spilled over in public. Arima was the first to make the point, but in the politest language. True, Monbusho's budget has been increasing, but the funds need to be spent selectively; there is "no need to give the same things to all universities at the same time". The universities have been slow to develop PhD programmes, and potential employers have been slow to appreciate the value of such developments.

Who are Japan's academics? Professor Robert Geller, a tenured professor of geophysics at the University of Tokyo, complains that there are not enough non-Japanese. He is one of only 25 tenured academics at Japanese national universities, out of a total teaching staff of 35,000, who are not Japanese nationals. (There are ten times as many untenured academic staff from overseas.)

Geller wants more, not to provide jobs for people unemployed elsewhere, but for the sake of the Japanese universities themselves. Inbreeding is a problem. At the University of Tokyo, 86.8 per cent of academics are graduates of the same university. The percentage of teachers at the

University of Kyoto who graduated there is smaller, at 80 per cent. Elsewhere in Japan, the inbreeding index is more like 50 per cent.

The explanation of that phenomenon was agreed by Geller and Sukeyasu Yamomoto, professor of physics at Sophia University (whose PhD is from Yale): appointments to academic vacancies are made by groups of academics working in a particular field, then automatically approved by the department and the faculty to which the group belongs — and almost never questioned by Monbusho.

The result is what Yamomoto called "selfishness and irresponsibility". Faced with the need to fill a vacancy, academics think first of their own students, often then of their chums. "These are strong words, I know," Yamomoto said last week, "but I believe them to be true." One ironic result of the recent pressure on university funds, which requires a 3 per cent reduction of faculty numbers over five years, is that professors continue to be appointed "at the expense of [the numbers] of junior faculty".

Yamomoto also said last week that academic freedom in Japan is essentially a sham. Professors are indeed free to choose the fields in which they work, with the consequence that an engineering department with "100 professors may have 100 laboratories", but the "total dependence" of all academics on Monbusho (for approval as well as funds) means that "it is virtually impossible to behave in defiance of Monbusho", yet there is "no such thing as accountability, only irresponsibility". Yet the only freedom that professors can be sure of is "the freedom not to be sacked".

The root of Monbusho's power was identified by Shinichi Yamamoto of Tsukuba University, whose task is the study of the modern university. This Yamamoto may well be a means by which Dr Leo Esaki, the man who gave IBM the Esaki diode before returning to be the president of Tsukuba, seeks to deflect some of Monbusho's influence.

His explanation of the ministry's power is the practice by which the ministry supplies the administrative heads of universities. The crucial post is that of secretary-general, but registrars and financial administrators are also appointed in this way. "We have no profession of independent university administrators", said Yamamoto.

Esaki, chairman of the panel discussion in which this plain-speaking bubbled up, plainly relished his position. After one outspoken criticism of the ministry, he declared that "If there are members of Monbusho in the audience" [there were], "I'll give them time to answer." He paused just long enough to let people sense the challenge had gone by default.

John Maddox

Conditions for excellence

The conference agreed to a statement previously discussed in detail by the speakers, and whose chief points are as follows:

Cultural, linguistic and social differences require that university systems differ among themselves, but there are common principles that should assist in the cultivation of excellence.

First, excellence should be the primary criterion in decisions on appointments and funding.

Second, excellence in research is not an excuse for mediocrity in teaching.

Third, regular and objective assessment of research and teaching is essential.

Fourth, flexibility (of institutions and departments) is essential in responding to

changing circumstances and in seizing research opportunities (especially across disciplines).

Fifth, an institution or department largely relying on internal appointments is unlikely "to become or remain" a centre of excellence. "As far as possible", foreign staff should be treated on the same terms as nationals.

Sixth, networking internationally is crucial.

Seventh, institutions need to be free to decide how the bulk of their income is to be spent on research and teaching, with external funding bodies interfering only to the extent required to ensure that public money is properly spent.

Copies of the full report are available on request.

Deep earthquakes shake up debate

Heidi Houston

THIS has been a bumper year for deep earthquakes. The magnitude 7.6 earthquake beneath Tonga on 9 March enjoyed a brief spell as the largest in 24 years, but was overshadowed on 9 June by a deep earthquake of magnitude 8.3 in Bolivia, which released ten times as much seismic moment and is the largest ever recorded. The seismic records are of unprecedented quality. In addition to global networks, in each case a temporary array of broadband seismometers had by coincidence been deployed nearby — indeed, participants at a meeting this month* were half-inclined to suspect that those responsible for the deployment knew something the rest of us don't about earthquake prediction. How such deep failure occurs has long been an enigma, with a welter of different mechanisms proposed. In recent years transformational faulting has been gaining favour, but now it seems that the rupture processes for these earthquakes are not quite what were expected.

Deep earthquakes, although similar to shallow ones in their primarily double-couple (shearing) nature, are thought to proceed through a different mechanism. The frictional mechanisms that produce shallow earthquakes are strongly inhibited by increasing pressure and temperature at depth; crack opening seems improbable at such high pressures. The observed properties of deep earthquakes do seem to differ from those of shallow events: aftershocks are much less commonly detected¹, and seismic waveforms reveal subtle differences in the rupture properties of the events²⁻⁴. Because aftershocks are usually the most reliable indicator of the main fault plane, for deep events it is often difficult to determine where the fault lies.

The differences led to the proposal about five years ago to the proposal about five years ago of an alternative mechanism for the generation of deep seismicity, based on laboratory experiments in which a shear instability can be produced by the runaway transformation of metastable olivine to spinel, with a consequent reduction in volume⁵⁻⁷. The process is analogous to brittle failure, but involves interaction and coalescence of spinel-filled 'anticracks' rather than dilatational microcracks, and thus can operate under great pressure. The idea is that metastable olivine could persist in the centre of a subducting slab while the temperature remained below a critical value (about 600 °C; ref. 8), but would transform on further heating, with a positive feedback as the transformation is

exothermic. So the fault plane of the mainshock of a deep earthquake should lie within the cold core of a slab, inside the critical isotherm. Many aspects of deep seismicity have been found to be consistent with such faulting^{5,7}, but details of deep faults remained sparse.

Then came the Tonga earthquake, and here at last there was no shortage of information: the nearby regional array of seismometers picked up more than 80 aftershocks⁹. Evidently global networks have been missing at least some aftershocks because of their low magnitude (this point is still more vividly demonstrated by the aftershock sequence of the Bolivian quake). The overall pattern of the Tonga event looks oddly like that expected for a shallow earthquake of similar magnitude, with just as many aftershocks and a decay rate typical of shallow events (D. A. Wiens, Washington Univ.).

The aftershocks lie on a near-vertical plane about 560 km deep and similar in orientation to one of the two possible planes for the mainshock rupture. The plane cuts across the slab and extends outside the region of background seismicity. A logical inference, supported by analysis of mainshock waveforms from the regional array, is that the mainshock slip occurred on this plane (J. J. McGuire, Washington Univ.), although there is conflicting evidence in the distantly recorded body waves for slip on the other plane (M. Antolik, Univ. California, Berkeley; S. Goes, Univ. California, Santa Cruz). If, as is generally the case for shallow earthquakes, the aftershocks do delineate the fault plane, the broad rupture area (50 km × 65 km) suggests that faulting can propagate outside the cold core of the slab, and that aftershocks too can occur outside the core.

For the Bolivian earthquake the situation is rather different. The body waveforms suggest that the three or four slip episodes making up the mainshock all lie about 630 km down but are separated horizontally by about 40 km. The simplest inference, backed up by waveform analysis both including the regional data (S. Beck, Univ. Arizona) and excluding it (ref. 10; W.-P. Chen, Univ. Illinois; Antolik; Goes), is that slip occurred on the horizontal nodal plane. The aftershocks, which could only be seen in data from the regional array, are smaller and less numerous (remarkably, only one had a magnitude larger than 5.0, compared to 11 such aftershocks for the Tonga quake). They apparently do not define the fault plane but lie in a diffuse zone dipping 45° (S. Myers, Univ. Arizona) — presumably

along the slab, but its orientation is not well established in this region. To my eye, the aftershocks fall into two groups, those lying on the inferred mainshock fault plane, and those in a dipping zone no more than 15 km wide following the centre of the slab.

How easily does this sit with the transformational faulting model? The extent and orientation of the mainshock fault planes for both quakes cannot be entirely contained within the critical isotherm, given simple models for the thermal structure of the subducting slab. But it does seem that no aftershock definitely occurred both off the mainshock fault plane and far from the cold centre of the slab.

At the meeting, controversy centred on whether the anticrack mechanism must be modified or rejected in light of these new results. The simplest such models cannot account for mainshock slip outside the metastable zone, but it may be possible for slip to be spread outside the core of the slab by the forces associated with a large earthquake (S. Kirby, US Geological Survey; H. Green, Univ. California, Riverside) or by shear melting during faulting (H. Kanamori, California Institute of Technology). However, it seems it is also necessary to nucleate some aftershocks on the mainshock fault plane where it extends outside the metastable zone. No one quite knows how, but a transient, localized recrystallization of spinel (Kirby) or metastability of enstatite (ref. 11; McGuire) were suggested.

A minority maintained that alternatives to the anticrack mechanism must be sought. They suggested that faults formed at the Earth's surface as the slab begins to subduct could be reactivated at great depths if sufficient fluid were released from hydrated minerals in the fault zone to lubricate the fault and allow slip (P. G. Silver and C. Meade, Carnegie Institution of Washington). The orientation of the Bolivian fault plane is consistent with this possibility but the Tonga fault plane is perpendicular to the subduction trench and extends about 60 km into the slab as well. It is hard to see how water could penetrate so deep into the slab during the faulting at the surface, and thus it is doubtful that the Tonga earthquake

*Fall Meeting of the American Geophysical Union, San Francisco, 5–9 December 1994.

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occurred on a reactivated fault (McGuire), although lineations in seismicity which seem suggestive of reactivated features do occur in the vicinity¹². A further difficulty for the pre-existing fault hypothesis is that it seems inconsistent with the size distribution of deep events in South America. There is an unusually high ratio of large to small earthquakes in the deep portion of the subduction zone, but not at shallow or intermediate depths — hard to explain if all stem from the same population of faults.

The problem of fitting large deep earthquakes into a narrowing cold zone applies to most thermally controlled mechanisms, afflicting not only transformational fault-

ing but the pre-existing fault hypothesis as well. Preservation of metastable olivine and the stability of hydrous phases are largely governed by thermal state.

So, despite the new and striking observations, the physical mechanism that allows faults to slip deep beneath the Earth's surface looks likely to remain controversial for some time. Reactivation of shallow faults so far seems far-fetched, but reactivation of the debate has certainly been achieved. □

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The new findings add important information on how mechanisms of nitrate retention are affected by chronic nitrogen deposition.

Durka *et al.* show that, even in sites with healthy forest growth, a large fraction (16–20 per cent) of atmospherically deposited nitrate moves through the forest ecosystem entirely without interacting with potential retention mechanisms. This result provides strong support for a hypothesis developed by European and North American research groups^{5–10} which predicts that saturation of nitrogen retention mechanisms, coupled with hydrological losses of nitrate, is a first step towards a fundamental change in the nitrogen cycle of forests that are exposed to chronic nitrogen deposition.

Durka *et al.* go one step further by evaluating how the nitrogen cycle has changed during the critical transition from healthy forest growth to forest decline. They show a substantial reduction, in one case a complete loss, in the capacity for declining forests to retain atmospheric inputs of nitrate. Moreover, internal production of nitrate remained high in declining forests, causing further acidification due to liberation of acidity during microbial nitrification.

The environmentally unsettling implication of these results is that forest decline might be a self-accelerating process in which initial forest damage is associated with reduced retention of nitrate which, in turn, accelerates any negative effects on the forest ecosystem caused by elevated nitrate. If so, forest response might not occur gradually, but

NITROGEN CYCLE

Stable isotopes, unstable forest

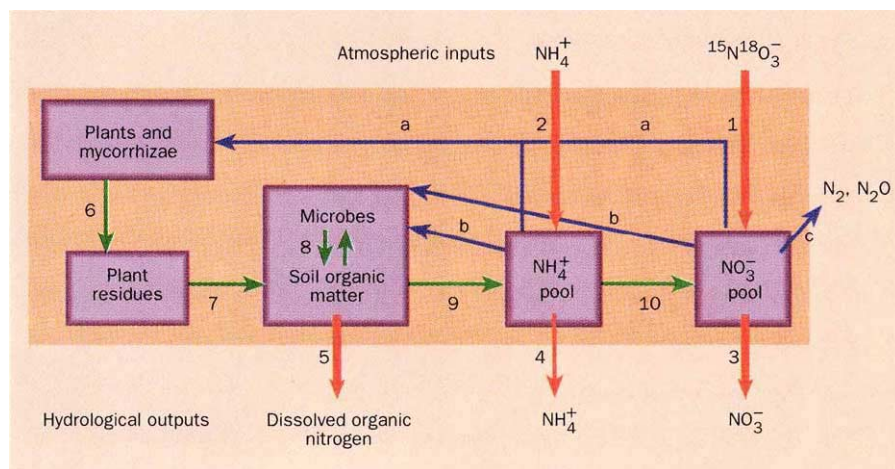
Lars O. Hedin

As a consequence of human industrial and agricultural activities, temperate forests in regions of Europe and North America have been exposed to unprecedented rates of atmospheric deposition of nitrogen, sulphur and acidity since the end of the last century. Nitrogen is critically important to forest nutrition, so has this chronic deposition altered natural patterns of nitrogen cycling within temperate forest ecosystems? Detailed mechanistic understanding has been difficult to come by, in part because we lack a direct method of tracing how atmospherically deposited nitrogen interacts with the complex nitrogen cycle of forest ecosystems (see figure). On page 765 of this issue, Durka *et al.*¹ remedy this omission by using the natural isotope abundances of nitrogen and oxygen to follow the fate of atmospheric nitrate through healthy and declining Norway spruce forests in Germany. Their results show that the onset of forest decline is associated with a shift in the nitrogen cycle characterized by a dramatic drop in the ability of microbial and plant communities to buffer the potentially harmful effects of nitrate.

Natural abundance isotope analysis of $\delta^{15}\text{N}$ is, by itself, not an ideal tool for tracing nitrate in forest ecosystems because of the relatively narrow range of isotopic differences between alternative sources¹. To improve their ability to trace nitrate, Durka and colleagues take advantage of the fact that the $\delta^{18}\text{O}$ value of anthropogenic atmospheric nitrate (path 1 of the figure) differs substantially from nitrate that is produced internally within the soil from microbial nitrification (path 10). ($\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ are defined in the figure legend.) This isotope information allows them to calculate the degree to which the forest nitrogen cycle is able to buffer atmospheric nitrate inputs by the

retention mechanisms of plant and microbial uptake, denitrification and abiotic immobilization² (paths a, b and c).

These retention mechanisms are critical in determining what impact nitrogen deposition has on forest ecosystems, and on the export of nitrogen to downstream aquatic ecosystems². Retention of nitrate is of particular importance, as nitrate is more mobile than ammonium in temperate zone soils (compare paths 3 and 4), and can contribute to depletion of nutritionally important base cations in soils³, to aluminium mobilization² and to acidification of soils and surface waters⁴.



Simplified model of the nitrogen cycle in forest ecosystems, with emphasis on dominant processes identified by Durka *et al.*¹. Red arrows indicate inputs and outputs of nitrogen: atmospheric deposition of nitrate (1) and ammonium (2); hydrological loss of nitrate (3), ammonium (4) and dissolved organic nitrogen (5). Blue arrows indicate mechanisms of inorganic nitrogen retention: uptake by plants and mycorrhizae (a); uptake by microbial communities (b) or abiotic immobilization (b) into soil organic fraction; microbial denitrification to N_2 and N_2O (c). Green arrows indicate nitrogen transformations associated with plant-derived organic matter with high initial carbon to nitrogen ratio: litter fall, root turnover and root exudates (6); incorporation of plant residue nitrogen into soil-microbial complex during humification (7); microbial decomposition of soil organic matter (8); microbial regeneration of inorganic nitrogen by ammonification (9); oxidation of ammonium to nitrate by microbial nitrification (10). ($\delta^{15}\text{N} = [({}^{15}\text{N}/{}^{14}\text{N})_{\text{sample}}/({}^{15}\text{N}/{}^{14}\text{N})_{\text{standard}}] - 1$; $\delta^{18}\text{O} = [({}^{18}\text{O}/{}^{16}\text{O})_{\text{sample}}/({}^{18}\text{O}/{}^{16}\text{O})_{\text{standard}}] - 1$.)

as a sudden shift in the nitrogen cycle.

What causes the observed deterioration of nitrate retention in declining sites? With limited demand from vegetation (path a) because of forest decline, the internal nitrogen cycle is driven by microbial consumption of inorganic nitrogen (path b) balanced by production from ammonification and nitrification (paths 9 and 10). Microbial consumption of inorganic nitrogen can be substantial², reflecting nitrogen limitation during decomposition of organic matter with high carbon to nitrogen ratios (path 8). So in the absence of any direct inhibition of microbial uptake by nitrate or acidity it is plausible that reduction in nitrate retention is caused by the release of microbial decomposers from nitrogen limitation. Possible mechanisms for such release include reduced production of labile plant residues with high carbon to nitrogen ratios (litter, roots or root exudates) in declining forests (path 6), and a progressive narrowing of carbon to nitrogen ratios of soil organic matter due to abundant supply of inorganic nitrogen¹¹.

There is evidence that some forest areas in Europe and North America are close to nitrogen saturation, and that these forests respond to increased nitrogen deposition in a manner that is qualitatively consistent with the results of Durka *et al.* For example, leaching losses of nitrate occurred immediately, even at low rates of experimental nitrogen addition (15–31 kg N ha⁻¹ yr⁻¹) to high-elevation spruce-fir stands¹², and a beech-dominated watershed¹³ in the northeastern United States. Additions to Swedish beech forests¹⁴ (60–180 kg N ha⁻¹ yr⁻¹) induced leaching of nitrate and base cations, and additions to Swedish spruce watersheds¹⁵ (30–50 kg N ha⁻¹ yr⁻¹) induced winter nitrate losses.

In contrast, Aber *et al.*¹⁶ found that red pine and oak-maple stands retained most of the nitrogen added over the first three years of treatment, even at high applica-

tion rates (150 kg N ha⁻¹ yr⁻¹). Such variable response among forests points to the need to consider community composition and, in particular, stand age as additional determinants of nitrogen retention. Biogeochemical theory predicts that mature old-growth forests, because of their low rates of net biomass and nutrient accumulation¹⁷, ought to be particularly sensitive to chronic nitrogen inputs¹⁸. In fact, comparisons of unpolluted and polluted old-growth temperate forests show great differences in hydrological losses of

nitrate and ammonium as a function of atmospheric nitrogen deposition¹⁸. The large areas of European and North American forests that currently are being built up, owing to disturbances during the last century and the early half of this century¹⁹, are therefore likely to become progressively more sensitive to chronic nitrogen deposition as they mature. □

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SIGNAL TRANSDUCTION

Look at a tyrosine kinase

Tony Pawson

THE structure described on page 746 of this issue¹ by Hubbard and colleagues will attract the attention of a wide constituency of biologists, as well as scientifically minded clinicians. The authors have solved the three-dimensional structure of the human insulin-receptor tyrosine kinase domain, and it tells us something not only about how insulin works but about tyrosine kinases more generally.

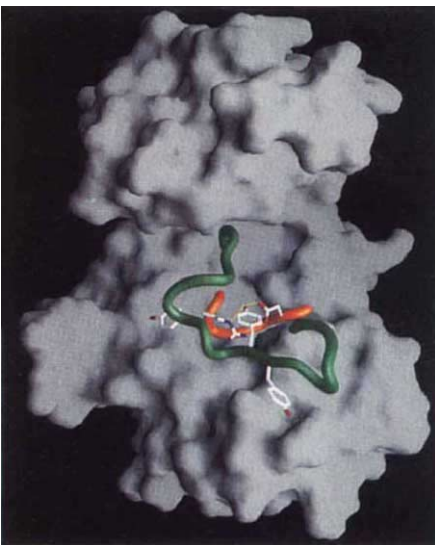
Since the purification of insulin in the early part of this century, and the demonstration that it could often alleviate diabetes, this remarkable molecule has served as a guide for those interested in

cellular signalling and metabolic disease. Like other hormones, insulin has no intrinsic ability to modify cell function, but must exert its effects by binding to a specific receptor that spans the plasma membrane and possesses a cytoplasmic domain with protein-tyrosine kinase activity^{2,3}. The activated receptor unleashes a series of biochemical signalling pathways within the cell that stimulate intracellular metabolism, gene expression and DNA synthesis⁴. The importance of this receptor to insulin's physiological activity is underlined by loss-of-function mutations within the insulin-receptor (IR) gene. These lead to a form of diabetes that is relatively resistant to insulin treatment⁵, which is to be expected if the aberration lies not in the production of functional insulin but in a molecule required for insulin to deliver its biological signal.

In themselves, these are good reasons why the biochemical function and molecular structure of the IR are of the greatest interest. But the IR is also a representative of a large family of receptor tyrosine kinases that control many aspects of cell growth and differentiation in both the embryo and adult⁶. Furthermore, dominant activating mutations in tyrosine kinase genes can transform their previously docile products into aggressive oncoproteins, capable of inducing human malignancies⁷. So information about the IR catalytic region is of more general significance, because all tyrosine kinase domains are closely related at the level of primary amino-acid sequence, and are therefore likely to have many structural and regulatory features in common⁸.

What, then, do tyrosine kinases look like, how are they converted from a repressed to an active state, and how do they discriminate tyrosine as a substrate from the other hydroxyamino acids serine and threonine? Hubbard *et al.* now provide the first answers to these questions.

The binding of ligands to receptor tyrosine kinases usually induces the



Molecular surface of the tyrosine kinase domain of the human insulin receptor with the catalytic loop (orange) and the activation loop (green) highlighted. The activation loop contains three tyrosine residues which are autophosphorylated in response to insulin. In the non-phosphorylated, inactive form shown here, one of these tyrosines, Tyr 1,162, is bound in the active site of the kinase, acting as an autoinhibitor. The side-chain hydroxyl group of Tyr 1,162 is hydrogen-bonded (yellow dotted lines) to conserved residues Asp 1,132 (the catalytic base) and Arg 1,136 in the catalytic loop.

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monomeric receptors to oligomerize in the plane of the membrane, and to autophosphorylate through an intermolecular reaction⁶. Autophosphorylation on tyrosine residues within the catalytic domain is associated with enzymatic activation, while phosphorylation in non-catalytic regions of the cytoplasmic domain can provide docking sites for downstream signalling proteins with Src homology (SH)2 domains⁹.

The IR is unusual in several respects. It is a heterotetramer, composed of two external α -chains disulphide-bonded to β -chains that cross the membrane and possess an intracellular tyrosine kinase domain^{2,3}. Insulin-binding presumably induces a conformational change in the receptor that juxtaposes the two β -chains, allowing for phosphorylation in *trans*. Activation of IR tyrosine kinase activity depends on autophosphorylation of three neighbouring tyrosine residues, located in the heart of the kinase domain¹⁰. Finally, the receptor shows little in the way of direct SH2-binding sites, but rather phosphorylates proteins such as IR substrate-1 that function as SH2-docking proteins⁴. Hubbard *et al.* have crystallized a 306-residue stretch of the IR containing the entire catalytic domain, which proves to be ideal for addressing the fundamental issues raised above. The resulting structure is both beautiful and informative.

The crystallized IR tyrosine kinase domain is in the unphosphorylated, inactive form. Its overall structure is similar to those of previously analysed protein-serine/threonine kinases, such as the cyclic-AMP-dependent protein kinase (PKA, or cAPK) catalytic subunit, in the sense that it has a small amino-terminal lobe (presumed to bind ATP) connected by a single strand to a large lobe containing the principal elements of the active site and the peptide binding site¹¹. Unlike the active form of PKA, where these two lobes are closely apposed, the two lobes of the inactive IR tyrosine kinase domain are rotated away from one another, in a fashion that would probably prevent productive interaction of residues involved in ATP-binding with those mediating catalysis.

But the most marked difference between PKA and the IR is in an extended sequence in the large lobe, referred to as the activation loop. In the IR, this loop contains the three tyrosines that become autophosphorylated during receptor activation. Strikingly, one of these residues, Tyr 1,162 actually sits in the active site (see figure), and is hydrogen-bonded to the catalytic base, Asp 1,132. A network of interactions involving residues in the activation loop hold Tyr 1,162 in a position that blocks access to the active site. Why, if there is a tyrosine in the active site, does it not automatically become phosphorylated? The answer seems

to be that residues in the activation loop of the unphosphorylated IR occupy the same space that would otherwise contain bound ATP. Hence the insertion of Tyr 1,162 into the active site not only excludes the entry of exogenous substrates, but precludes ATP-binding, and as a consequence represses kinase activity.

Hubbard *et al.* propose the following scheme to account for receptor activation. Ligand binding is postulated to move the kinase domains of the heterotetrameric receptor close together, and thereby allows transient cross-phosphorylation of Tyr 1,162 and adjacent tyrosines in the activation loop. Upon phosphorylation, Tyr 1,162 is probably displaced from the active site, and the phosphorylated activation loop is presumably stabilized in a new conformation. The upshot of these changes is to allow the binding of ATP and exogenous peptide substrate, and thereby greatly stimulate catalytic activity. This proposition is consistent with most of the relevant biochemical and mutagenesis data.

Pleasingly, all tyrosine kinases contain at least one tyrosine in the activation loop at a position corresponding to Tyr 1,162 (ref. 8). In several cases this tyrosine is a bona fide intermolecular autophosphorylation site, important for the activation of wild-type tyrosine kinases and the transforming activity of their oncogenic counterparts¹². The preliminary scheme outlined for the IR is therefore likely to

hold for many tyrosine kinases. The structure has many other delights of interest to protein kinase aficionados, most notably in hints as to how tyrosine kinases distinguish tyrosine from serine/threonine at the active site.

Finally, to return to diabetes. The effects of missense mutations and resulting amino-acid substitutions in the IR that cause insulin-resistant diabetes can now be understood at the atomic level, based on their anticipated effects on the structure of the IR tyrosine kinase domain — it is satisfying, and may be clinically useful, to be able to explain a human genetic disorder in terms of protein structure. □

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RNA PROCESSING

Splicing in space

Iain W. Mattaj

WHAT is the spatial relationship between the transcription of pre-messenger RNA and subsequent splicing? Two papers — one in *Journal of Cell Biology*¹, the other on page 809 of this issue² — contribute to the re-emergence of an idea formulated a decade ago by Fakan *et al.*³. Paradoxically, the model proposes that most splicing does not take place at sites in the nucleus where splicing factors are concentrated, but rather occurs close to, or at, sites of transcription.

Seen in the light microscope, most eukaryotic cell nuclei are relatively amorphous structures whose only major distinguishing features are nucleoli, the sites of ribosome assembly. The electron microscope and molecular probes^{3–6} have, however, revealed that specific DNAs, RNAs and proteins are concentrated in particular regions of the nucleus. These 'compartments' are not bounded by membranes and may well, like the nucleolus, be functional domains in which particular events, such as RNA transcription or DNA replication, take place. The intra-

nuclear distribution of components of the pre-mRNA splicing machinery, the proteins and small nuclear ribonucleoproteins (snRNPs) required for the removal of intervening sequences, has been intensively studied in the hope of answering the question posed above.

Early electron microscopic work^{3–6} identified three distinct RNP- or RNA-containing structural components of the nucleoplasm. Perichromatin fibrils (PFs) were largely found near chromatin, but also dispersed throughout the nucleoplasm. A minority were located adjacent to a second type of structure, formed by clusters of interchromatin granules (IGs). A small and variable number of coiled bodies (CBs), highly condensed structures found either in the nucleoplasm or, more rarely, on the surface of nucleoli, were also evident. In the light microscope, immunofluorescent probes for IGs, including antibodies that recognize either the SR family of splicing factors or snRNPs, give rise to a pattern of 20–50 bright 'speckles'. The same probes also

recognize PFs, producing faint staining of a diffuse reticulum that apparently joins the speckles. CB probes show a few intense foci of staining^{5,6}.

Nuclear pre-mRNA and mRNA have short half-lives. Autoradiography of nuclei whose RNA had been briefly pulse-labelled showed that the bulk of newly synthesized RNA polymerase II (pol II) transcripts were associated with PFs. The IGs and CBs were not labelled, leading to the conclusion that RNA associated with these structures is relatively stable and could not be solely composed of (pre-)mRNA⁴ (shorthand for pre-mRNA and mRNA). In contrast to (pre-)mRNA, protein and snRNP splicing factors are most highly concentrated in IGs and CBs (refs 3–6). The sites of highest splicing-factor concentration did not incorporate radioactivity in the pulse-labelling experiments. So — the argument ran — they could not be the sites at which most pre-mRNAs were spliced³. These ideas found support in accumulating biochemical evidence that splicing might occur largely, although not entirely, co-transcriptionally (see ref. 7 for review).

These apparently clear waters have become muddled over the past four years. For example, in *in situ* hybridization studies⁸ bulk nuclear poly(A)-containing RNA was found to colocalize with snRNPs and the SR splicing factor SC35 in speckles. Equating nuclear poly(A) with newly made (pre-)mRNA, the authors suggested that splicing would occur during passage through the speckles (that is, in IGs), a proposal that was incompatible with the pulse-labelling data^{3,4}. This contradiction has apparently now been resolved by Huang *et al.*¹, who have shown that a large amount of nuclear poly(A)-containing RNA, in particular that associated with IGs, does not disappear when pol II is inhibited. Given the short nuclear half-life of (pre-)mRNA, these experiments suggest that the poly(A) in IGs is by no means all (pre-)mRNA, but may instead represent a novel class of stable nuclear RNA. On the other hand, most of the PF-associated RNA did disappear on pol II inhibition, supporting the earlier idea that PFs contain (pre-)mRNA.

The next dent in the model of Fakan *et al.*³ came from the demonstration that specific nuclear (pre-)mRNAs are distributed nonrandomly with respect to IGs. In particular, *in situ* localization showed close proximity (in the case of *c-fos*) or a large degree of overlap (in the case of fibronectin¹⁰) between nuclear RNA transcripts and IGs. Enter Zhang *et al.*², who have now examined the distribution of newly synthesized cellular β -actin transcripts as well as adenoviral (pre-)mRNAs. As well as gene- and intron-specific probes, they used probes that only hybridize to mRNAs once splicing has occurred (see Fig. 1 page 809). This is an important

technical advance, as it allows definitive identification of spliced RNAs.

Zhang and colleagues draw two conclusions from their observations. First, within the resolution of the light microscope, in both the cellular and viral cases splicing occurs at the location of the gene. This implies that, as in several other examples⁷, splicing is likely to be cotranscriptional. Second, neither spliced mRNA nor intron sequences localize to regions where splicing factors are most concentrated (IGs and CBs). They are not excluded from these regions, but seem to be distributed randomly with respect to them.

These results are in excellent agreement with the pulse-labelling studies⁴, and so probably represent the general case which can be stated as follows. Transcription occurs at many sites in the nucleoplasm, sites that are probably randomly distributed with respect to IGs and CBs, and most splicing occurs at the point of transcription. Fibronectin and *c-fos* are exceptions, where transcription, and probably also splicing, for some reason occurs non-randomly with respect to IGs.

If IGs (and CBs) are not the sites of splicing, why do splicing factors collect there? Both structures are highly dynamic^{5,6}, leading to suggestions that they store excess splicing factors which can be mobilized to sites of pre-mRNA synthesis, or are places where factors that have been involved in one round of splicing are recycled for further use.

A paper published last year¹¹ provided evidence for the first possibility. The authors examined the localization of adenoviral DNA and RNA at various times after infection in relation to the location of snRNPs and the SC35 splicing factor, and concluded that adenoviral infection caused a reorientation of snRNPs and SC35 to sites of adenovirus transcription. As viral RNAs make up roughly half of total nuclear transcripts late in infection, this would indicate that snRNPs and SC35 might be depleted from the IG (and possibly CB) stores as they move to sites of active transcription and splicing.

These conclusions have, however, been questioned. First, two groups have now shown that the results obtained with the SC35 antibodies in adenoviral-infected cells were not valid. The antibody used cross-reacts with a major adenoviral protein, the DNA-binding protein (DBP). In infected cells, the supposed 'SC35' localization is actually that of DBP and not of the SR splicing factor^{2,12}. Second, the localization of snRNPs, SR splicing factors, adenoviral RNA and DBP during the course of adenovirus infection has been followed with a battery of specific probes^{2,12,13}. Although one of these studies¹² confirms the earlier conclusion¹¹ that snRNPs can be localized to sites of adenoviral pre-mRNA transcription dur-

ing the late infection stage, this appears to occur only in a minority of cells and only during a transient period^{12,13}. Importantly, this period is not the time at which late viral transcription is maximal, and thus does not correlate with the time at which splicing of viral transcripts is at its height.

These results do not support the idea that IGs are passive stores of splicing factors for use at times of high demand. Instead, they may suggest that the transient colocalization observed is a stage in the marked reorganization of the nucleus induced by adenoviral infection. Other viruses produce similar drastic effects on the morphology of IGs, and in at least one case, that of herpes simplex virus, IG reorganization is induced by a viral protein without any requirement for the production of intron-containing viral RNAs¹⁴.

An obvious question then arises. Why are splicing factors present in such abundance that they are mostly found at sites distant from those at which splicing is occurring, even at times when the requirement for splicing activity is extremely high (as in late-stage adenoviral infection)? A simple but speculative answer would be that if IGs and CBs are sites of splicing factor pre-assembly and/or recycling, and if these are rate-limiting steps *in vivo*, splicing factors would accumulate in IGs and CBs independent of the amount of splicing taking place. This would not exclude the possibility that IGs or CBs could also be sites of factor storage.

We are left with an ageing but newly strengthened model for nuclear transcript organization. We are also left with questions. What, for instance, are the organizational principles underlying IGs and CBs, and what causes the drastic morphological changes seen after viral infection, pol II inhibition and so on? More importantly still, what are the functions of IGs and CBs? Finally, is there a functional significance to the fact that some genes seem to be distributed nonrandomly with respect to IGs while others do not? □

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Venus after the flood

James W. Head III

ATTEMPTS to break the chains of narrow, religiously based interpretations dominated the early history of geological thought on the Earth. Natural events and features were interpreted to be the result of acts by a divine creator, and most observed geological deposits were linked to the Noachian flood. Slowly, an appreciation of the great age of the Earth melded with an increasing awareness that present geological processes, operating at relatively constant rates over extended geological time, could produce the range of features seen in the rock record. Catastrophism, as an explanation for geological events, fell out of favour, and uniformitarianism, the idea that the past history of the Earth could be interpreted by observing present processes and their rates and extending them back in time, came to dominate geological thinking.

Had such thinking arisen on Venus instead of the Earth, the situation might have been different. On the basis of global images and topography from NASA's spectacularly successful Magellan mission to Venus, it appears that there is no surface terrain dating from the first 85 per cent of the history of Venus. Planetary scientists have observed terrain that is largely volcanic plains, and the impact cratering record has been interpreted to indicate that a near-global tectonic deformation and volcanic flooding event occurred some 300–500 million years ago.

And that's not all. Apparently, not much has happened geologically since then. Neither the style nor the rates of geological processes are the same now as they were at the time of this global flooding event. Much analysis is now focusing on the nature of the geological activity in this post-flooding period. On page 756 of this issue, Price and Suppe¹ examine crater densities (related to the age of the surface) on specific well-defined features and structures, such as volcanoes and rift zones, and propose that they represent activity distinctly separated in time from, and unrelated to, the earlier widespread flooding events.

In earlier stages of investigation, before the Magellan pictures were available, Venus bore many similarities to other terrestrial planets, but the data were of mixed resolution, ranging over two orders of magnitude from several kilometres to hundreds. Images from Earth-based radio telescopes² and Soviet orbiting spacecraft³ showed evidence for extensive volcanic plains like those of the Moon and Mars. Earth-like linear mountain belts bordered patches of highly deformed and high-standing terrain, named tessera. Tessera

terrain bore similarities to continents on Earth. Broad rises featured spectacular branching rift zones and superimposed shield volcanoes, suggesting upwelling and hotspot volcanism. Linear discontinuities and symmetrical topographic profiles suggested the possible presence of spreading centres. But what were the main mechanisms of the transfer of internal heat across the outer thermal boundary layer, the lithosphere? Was it lateral

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Rare sight — an impact crater split by faulting on Venus.

movement, subduction and lithospheric plate recycling, as on the Earth, or conduction, as on the Moon, Mercury and Mars? Or could it be advection, the direct transfer of heat to the surface by volcanism at very high rates, as on Io, the innermost galilean satellite of Jupiter⁴?

As the Magellan high-resolution images rolled in strip by strip, a global picture emerged. The highly deformed and high-standing tessera terrain made up a little less than 10 per cent of the planet and was embayed by volcanic plains making up most of the rest of the surface. One might surmise that the ancient tessera dated from the first half of Venus's history and the plains represented subsequent long-term resurfacing either by vertical build-up (as on the Moon and Mars) or by lateral crustal spreading, as on the Earth.

But initial analysis of impact craters, their number, distribution and characteristics, told a different story^{5,6}. Instead of different crater densities for the tessera and various volcanic plains units, the crater distribution could not be distinguished from a spatially random population. Using the estimated flux of impac-

tors in the environs of Venus, observers interpreted the frequency distribution of crater sizes to mean that the crater retention age of the surface of Venus was about 300–500 million years. Even more intriguing was the fact that only a small percentage of the observed impact craters had been modified by subsequent volcanic and tectonic activity. Schaber and Strom and their colleagues interpreted this to mean that a catastrophic resurfacing event had erased all pre-existing craters some 300–500 million years ago, creating a new surface dominated by volcanic plains⁷. Following the catastrophic event, geological activity in the form of volcanism must have declined sharply, and this *tabula rasa* simply accumulated craters which remain largely unmodified to this day, hundreds of millions of years later.

Do such reduced rates of activity and changes in style reflect the waning stages of the great flooding event or do they represent a distinctly different period and style? In addressing this question, Price and Suppe¹ have analysed impact crater densities on various geological features, such as large volcanoes and tectonic rift zones, that are stratigraphically superposed on the widespread volcanic plains. Rather than looking at the distribution of craters on the planet as a whole, their approach is to examine crater densities on discrete features that might have formed from a single process (for example, shield volcanism or tectonic rifting). Mapping the global distribution of these features and then counting the craters on them, they propose that these terrains are sufficiently widely separated in time from the catastrophic resurfacing event that they represent separate, recent and ongoing geological activity (mean ages of 70–125 Myr), rather than the waning stages of the catastrophic event. Other workers⁸, looking only at volcanoes and features called coronae, find crater densities half the average density for the planet, implying an intermediate temporal transition of activity.

In one explanation for such a catastrophic event, periods of rapid plate tectonics are episodically interleaved with periods of lithospheric stability and relative quiescence, such as we see today⁹. In another explanation, the key lies in vertical crustal growth over geological time¹⁰. In this case, basalt is continuously extracted from the mantle and extruded to the surface layer upon layer, in contrast to the lateral formation and ultimate subduction of sea-floor basaltic crust on the Earth. On Venus, the thickening depleted mantle layer from which the basalts are extracted ultimately becomes gravitationally unstable and overturns, causing intense surface deformation. The overturn is accompanied by ascent of fresh mantle to shallow levels where it undergoes pressure-release melting, causing large-

NASA/Magellan Mission

scale volcanic resurfacing. This process is also predicted to be episodic, with a frequency of about 500 million years.

Could the young activity dated by Price and Suppe¹ be the beginning of the next phase of catastrophic evolution? Unfortunately, their data do not permit them to distinguish between continuing, steady activity, and the onset of a catastrophic event. Perhaps the high-resolution gravity data obtained just before Magellan's recent atmospheric re-entry and demise will address this question.

Marcel Proust described voyages of discovery as consisting of both seeking new landscapes and having new eyes. Planetary exploration in general and the Magellan results in particular have not only provided new landscapes, they have also made us think in new ways about the Earth. First came the emerging perspective that impact cratering is not an unusual and catastrophic event, but is uniformitarian in nature and a *likely* cause of biotic crisis in the Earth's history. Now we are

challenged to consider the possibility that such basic processes as plate tectonics may have been episodic, or even that the crust and upper mantle may have been catastrophically lost and renewed. Could this be a key to understanding the first half of Earth history? □

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CANCER

Sun protection factor p53

Alexander Kamb

THE stinging pain of serious sunburn is the immediate cost of careless exposure to the Sun. The deferred cost is skin carcinoma, one of the most common and most rapidly rising types of cancer.

On page 773 of this issue¹, Ziegler *et al.* show that a major target of the Sun's rays is the infamous tumour-suppressor gene *p53*, and the authors elaborate a plausible course of events to explain how *p53* mutations lead inexorably from repeated sunburns to skin cancer. It seems that sunburn, despite the pain, may actually be protective — a deliberate effort by skin to forestall the tumorigenic effects of ultraviolet light (UV).

One of the goals of cancer research has been to understand the process by which a normal cell evolves more malignant traits that culminate in invasive, metastatic neoplasia. The past several years have brought significant advances, perhaps most elegantly in the form of a model for colorectal-cancer progression developed by Vogelstein and colleagues². Tumour formation in the skin, as in the colon, has certain features that facilitate the study of tumour progression. Both organs are relatively accessible, and both have clearly defined stages of neoplasia. A further advantage of skin cancer as a model is that the initiating event, UV exposure, can be precisely controlled.

Skin cancer and colorectal cancer have in common the involvement of *p53*. Surprisingly, however, in skin *p53* seems to take part in some of the earliest events.

This runs counter to its role in colorectal cancer where mutations in the gene arise primarily after tumours have passed through the adenoma stage. The new analysis of tumorigenesis in the skin should dispel the misconception that *p53* is important only in the later stages of cancer — clearly the participation of *p53* depends on the specific type of cancer concerned.

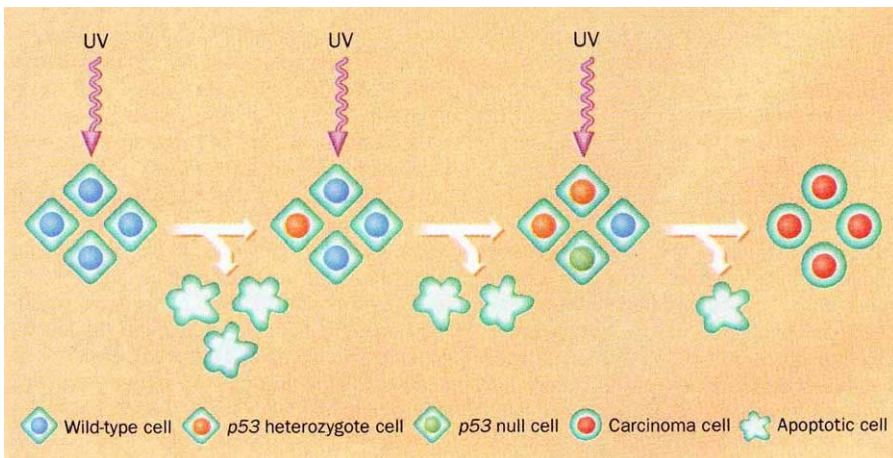
The *p53* gene seems to come into view whichever direction cancer research turns. It is subject to mutation in a multitude of cancer types, and in certain types mutation frequencies approach 90 per

cent³. A connection between UV and *p53* is not new. Irradiation with UV is known to arrest some cells during the G1 phase of the cell cycle in a *p53*-dependent manner^{4,5}. Cells that do not express functional *p53* protein are unable to arrest in G1 after irradiation, and proceed unchecked through DNA synthesis. This prevents the cells from repairing DNA damaged by UV, and results in a higher rate of mutation. The central role of *p53* in this process has earned it the name 'guardian of the genome'. Thus, one of the jobs of *p53* is to maintain tight control over the cell cycle to help preserve the integrity of the genetic material.

But *p53* has at least one other function. Under certain conditions, some cells adopt a different strategy in response to UV irradiation or other trauma^{6,7}. They commit suicide by initiating a set of steps culminating in cell death. This process, apoptosis, is believed to be a mechanism in metazoans for eliminating unwanted cells, including those that might eventually escape normal growth-control measures because they have incurred mutations. In mammals at least, *p53* has a key part in apoptosis and for this reason has been dubbed 'guardian of the tissue'.

Among other things, what Ziegler *et al.* have accomplished is to divert some of the attention on skin cancer away from the initiating event itself and onto the influence of *p53* as a guardian of skin tissue. It is beyond question that UV induces mutations in skin cells and that these lesions are critical in tumorigenesis⁸. But the apoptotic effects of UV may be equally important in development of skin cancer. When subjected to UV, skin cells can undergo apoptosis. This process gives rise to the swatches of skin that peel off several days after a severe sunburn. In the absence of *p53* function, however, the number of apoptotic sunburn cells is dramatically reduced.

What are the consequences of this al-



The dual effects of ultraviolet light on tumorigenesis in skin. Not only does UV induce mutations in genes such as *p53*, it also causes clonal expansion of *p53*-negative cells at the expense of normal cells, thereby increasing the number of cells that can spawn tumours. In effect, UV exerts a strong selective pressure in favour of cells deficient in *p53*.

teration in apoptosis? The authors point out that cells with diminished capacity to undergo apoptosis have a selective advantage. New episodes of Sun exposure gradually weed out the *p53*-positive, apoptosis-competent cells while the apoptosis-impaired cells survive and replace their normal counterparts. Thus, UV is doubly effective as a carcinogen (see figure).

But why is *p53* such a favoured target for mutation? There are several possibilities, one of which emerges from the work of Ziegler *et al.* They find that cells heterozygous for *p53* mutations (one mutant allele and one wild-type allele) are intermediate between wild-type and homozygous mutant cells with respect to apoptotic competence. This suggests that *p53* mutations are co-dominant for apoptosis; that is, a single inactivating mutation promotes survival of the heterozygote over the wild type.

A second explanation may be that, for the price of inactivating one gene, *p53* mutations have two important consequences; namely, deregulation of the cell cycle and reduced apoptosis. A third explanation is that somatic *p53* mutations may seldom or never compromise the viability of the cell, whereas mutations in other tumour-suppressor genes or oncogenes may do so. If most cancer genes can be altered only at specific times in tumour formation, and only in certain combinations without simply killing the cell, they may appear to have undergone mutation less frequently than *p53*.

Other issues are raised by the new work. Inactivation of *p53* does not completely abolish the cells' capacity to undergo apoptosis. What pathway (or pathways) is responsible for this residual apoptotic behaviour? Actinic keratoses, the precursors of squamous cell carcinomas, frequently contain *p53* mutations; yet these lesions regress spontaneously in the absence of UV exposure. Are cells with *p53* mutations at a selective disadvantage without UV? The skin, like the colon, is an attractive model in which to study cancer. In future studies, it will be important to extend to other tissues the concept of *p53* as a bona fide growth regulator highlighted by Ziegler and colleagues' informative analysis of skin tumorigenesis. □

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The continuing saga

Rodney S. Ruoff

CARBON nanotubes have attracted intense scrutiny since their discovery¹ in 1991. The empty core at the centre of a nanotube begs to be filled, and the low-melting-point metals lead and bismuth were induced several years ago to part-fill this centre core²; but the approach used has not been extended to high-melting-point metals, and the quantities of partially filled nanotubes produced were microscopic. Two considerable advances in the filling of the core volume in nanotubes with different metals have now been reported — one on page 761 of this issue³, the other a few weeks ago⁴. Guerret-Piécourt *et al.*³ have used direct synthesis in a direct-current (d.c.) carbon arc maintained in gaseous helium to which is added a metal. Tsang *et al.*⁴ treated nanotube material with nitric acid to open the tubes, with filling achieved from metal ions present in solution.

There are currently two types of nanotubes, different in appearance and in their mode of production. Multi-walled nanotubes occur inside a growth that builds up on the cathode of a d.c. carbon arc operated in a helium atmosphere¹. Single-walled nanotubes have been produced in the 'soot' that emanates from carbon arcs; these develop only when certain elements such as iron or cobalt are added to the carbon arc^{5,6}. Both Guerret-Piécourt *et al.* and Tsang *et al.* use multi-walled nanotubes; filling the single-walled variety remains on the horizon.

Using approaches similar to that on page 761, other groups have seen evidence of encapsulation of carbides of yttrium⁷ and gadolinium⁸ in nanotubes culled from the cathode growth from a typical d.c. arc. Although these results are tantalizing, the yield of partially filled nanotubes has been small, the encapsulate being a minor product alongside empty nanotubes, nested polyhedra which may be empty or partially filled, and other forms of carbon such as particles of graphite and amorphous material⁸. One might have asked, before this week's paper, whether direct production in the d.c. arc was really a suitable approach for filling carbon nanotubes with metals, although it has given high yields of filled or partially filled carbon polyhedra for certain elements^{9,10}.

But the new work shows that a variety of elements can partially fill nanotubes grown in such a way, and that some of the filled sections are around a micrometre in length. I suggest that a critical factor is the maintenance of an especially stable arc for long periods during the arc run, and that previous researchers who have not seen such yields were working with arcs that were not particularly stable. Smalley has

suggested that empty nanotubes grow on the cathode in a d.c. carbon arc (no metal present) because of very large electric fields at their tips, which serve to keep the tips open as carbon cation species are 'guided' to the open tips along the field lines¹¹.

Achievement of particularly stable arcs (uniform and non-fluctuating electric field at the cathode surface) is in a sense a defeat of the 'cathode spot' phenomenon, where the electric field is concentrated particularly on one spot on the cathode for a short time, and then hops to a different spot; this is deleterious to uniform growth of nanotubes. Videotapes taken in our laboratory clearly show that the current path in a typical d.c. arc hops back and forth over the cathode and anode carbon electrode surfaces. Active feedback in controlling the current and voltage of the arc can hold an arc in stable operation¹². An alternative method, initiating a glow discharge between graphite electrodes with a tesla coil coupled to a tungsten wire held close to the electrode pair, allows a stable current to be maintained and improves nanotube yields¹³. The yields of filled nanotubes in ref. 3 are in the 1–5 per cent range as estimated from transmission electron micrographs. If this method were to be combined with deliberate design of very stable arcs or glow discharges, it should be possible to improve yields and to study in detail the mechanism for filling.

Typical multi-walled nanotubes as currently produced are closed off or capped at the ends. The positive curvature of the end closure is achieved by pentagon insertion into the hexagonal bonding network, and the pentagon locations are probably more reactive. Proof of a faster rate of oxidation, and subsequent removal of the pentagons and capped portion, comes from the work of Tsang *et al.*⁴ who report a simple chemical method for opening nanotubes in a selective manner with boiling concentrated nitric acid. One can then imagine filling them with different solutions and depositing the solute of interest. Solvent removal could leave a residue which might itself be a metal, or on further treatment be converted to a metal.

An important aspect of solution-based filling is that a wide variety of compounds could probably be incorporated. For example, it is difficult to imagine *in situ* arc production of filled nanotubes with encapsulated NaCl or CdSe crystals, but solution-based filling should be possible. Control of encapsulate stoichiometry should be much greater with precipitation from solution than by arc methods. Be-

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cause the nitric acid treatment can be scaled up, the availability of open nanotubes is limited only by the availability of the capped feedstock. Ebbesen and co-workers, and Hwang, have also obtained good tube-opening results with a solution of potassium permanganate in acidic solution (T. Ebbesen, K. C. Hwang, personal communication).

Selective removal of tube tips by nitric acid shows that the pentagon carbon atoms and the carbon atoms under strain at the tips are reactive but that the hexagonal network of sp^2 -bonded carbon on the tube sides is not, on the timescale of treatment used in ref. 4. The ability of nitric acid to chew selectively along a line of pentagons, 'drilling' inwards through a series of corners in a nested polyhedral particle (R. S. R. and S. Subramoney, unpublished results) without destroying the hexagonal sheets, may lead to new recipes for carbon-coated nanocrystals where the nanocrystals are formed by techniques analogous to those used by Tsang *et al.* to fill nanotubes.

One aspect of ref. 4 deserves special scrutiny. One could infer from the procedures used that the final volume filling of NiO on conversion of a nickel precursor is determined by the concentration of nickel nitrate solution used. Straightforward calculation shows that a volume filling of only 0.27 per cent would result. But the lengths given as typical are 20 nm for NiO crystallites and 200 nm for nanotubes and the NiO crystallite fills the nanotube essentially completely over its 20-nm length⁴. So the filling cannot simply be a question of the bulk solution concentration. The roughly 10 per cent filling with NiO means that the nickel nitrate precursor (a solid) must essentially completely fill the tube (taking account of molecular weights and respective densities). How does nearly 40 times 'too much' NiO end up, following calcination, in the nanotube, or how could the tube be filled completely with a solid precursor? I leave it to the reader to ponder the implications of precipitation of solids inside the nanotubes when the bulk solution outside them is subsaturated.

We may speculate about possibilities that filling from solution can offer. One may think of the internal volume of a nanotube as a test tube for studying chemical reactions, or the thermodynamics or kinetics of phase transitions, in small spaces. For an ionic solution, is the concentration of ions in the solution *inside* the tube the same as in the bulk solution, or do unusual concentration gradients develop? If one forces a non-wetting liquid¹⁴ such as mercury into a tube with high pressure, will it stay in the tube when pressure is released? If so, and the filled tube is cooled to below the superconducting transition temperature of bulk Hg (about 4 K), perhaps interesting phenomena will be observed. At room temperature, will en-

capsulated Hg be a liquid? For the chemist chef who desires peas-in-a-pod, can opened nanotubes be filled with C_{60} ?

One wonders, too, how enclosed nanocrystals whose expansion is limited radially will melt. Topological constraints may govern phase transitions; for instance, preliminary theoretical efforts show that argon will have difficulty forming a complete solid phase inside some tube geometries even though the temperature is below the bulk freezing value, because of the difficulty of growing a non-frustrated cylindrical crystal inwards from the inner tube perimeter to the centre (M. Maddox and K. E. Gubbins, personal communication). The dynamics of water or of a long-chain polymer inside nanotubes could be fascinating and could be studied by NMR.

One wants to believe in quantum wires based on long nanotubes that have been completely filled with conducting metals, and progress in improving yield and quality of nanotubes continues at a good rate. But in my opinion this is a more distant goal than immediate studies of incorporating materials of relatively short length into opened nanotubes, and investigating their various properties. Progress in fundamental areas and in applications that do not rely on perfect filling should come first, such as studies of the thermodynamics and dynamics of solutions or solids inside nanotubes, and new catalysts.

But if we may dream of nanowires, how about taking a scanning probe microscope nanotip that is a carbon nanotube, clipping off its end by a wet chemical etch, and dipping it into an appropriate solution to produce a magnetic encapsulate such as Fe or Ni at the tip end? Perhaps such a tip could detect atom-specific magnetic resonance of biomolecules on surfaces. □

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Celestial graffiti

LAST week Daedalus proposed the scanning electron telescope. It was an orbiting launcher for strong electron and positive-ion beams (both together, to maintain overall charge-neutrality). The beams were to be aimed at planetary targets, so that earthbound telescopes could study the luminosity of their impact. He now proposes aiming the beams down towards the Earth.

When high-energy particles hit the high atmosphere, they excite an auroral glow. The shifting, filmy, magical curtains of the polar aurora are produced by the impact of solar-wind particles on the air molecules about 150 kilometres up. The molecules luminesce as they decay back to their ground state. Aurorae can have many colours: red (the 'proton glow' from protons in the solar wind), and yellow, green and violet from various spectral bands of electron-excited nitrogen and oxygen molecules.

A powerful, narrow particle beam directed from above should create a very bright 'point-aurora' at its site of impact. The colour and intensity of the auroral glow could be controlled by varying the energy and particle density of the beam. The positive and negative beams would create point-aurorae of different colours, which could be scanned independently around the sky. The application is obvious: celestial colour television. From space, it should be possible to scan a picture over the atmosphere 100 kilometres across and 150 kilometres up, and visible over an area the size of central Europe.

In the daytime, the brightest auroral picture would be imperceptible. Celestial television will come into its own at night. Low-orbit satellites will speed across the heavens trailing huge pictures in their wake, like publicity banners behind an invisible plane. Geosynchronous satellites will paint a stable frame that will fill the same chunk of sky night after night. At first Daedalus hoped for educational programmes, with leading astronomers pointing out planets and constellations to the upgazing masses. But the dream soon faded. He now expects the sky to fill up rapidly with the usual trash, advertisements and all. One consolation is that the pictures would be silent. Aurorae make no noise of their own, and even the radio emission from particle impact would be hard to modulate into a useful soundtrack. Furthermore, the Earth's ever-changing magnetic field will sabotage the line-scan, making the images wobble unsteadily. With luck this undignified wavering will discourage the more pompous nonsense, leaving the sky to the likes of Tom and Jerry.

David Jones

Regulation of *BRCA1*

SIR — *BRCA1*, a gene conferring susceptibility to early onset familial breast and ovarian cancer, has recently been cloned¹. We have found that the 5' end of *BRCA1* lies head to head with the 5' end of the *LA1-3B* gene², with a maximum distance of 295 base pairs (bp) between their putative first exons. This raises the possibility

BRCA1, and the two genes are divergently expressed⁴.

The genomic sequence at the 5' end of the *LA1-3B* gene was cloned and partially sequenced. This sequence contained the previously detected first exon of *LA1-3B* (ref. 2) as well as 800 bp of new sequence upstream. Comparison of this sequence

the database revealed 100% identity to a 131 bp region, 600 bp 5' to our published first exon, with a randomly isolated messenger RNA from the myeloblast cell-line KG1, termed ORF (N. Nomura *et al.*, unpublished results; accession number D30756). ORF also contained sequences identical to exons 2–19 of *LA1-3B*, but not the previously published exon 1 (here named exon 1B). This suggests that an alternative 5' exon (here named exon 1A), not present in the original *LA1-3B* cDNA clone, is expressed in KG1 cells. This provides evidence for alternative 5' starts of transcription in the *LA1-3B* gene.

Southern analysis of *BRCA1*- and *LA1-3B*-containing PAC and cosmid clones⁴ revealed that the putative first two exons of *BRCA1* and the 5' region of *LA1-3B* mentioned above (as well as a portion of the *LA1-3B* DNA data not shown) all reside on a single 3-kilobase (kb) *Pst*I fragment. Polymerase chain reaction (PCR) analysis detected a single 750-bp fragment, with the primer pair 1R and LH3 (see figure). This indicated that *BRCA1* exon 1 is centromeric to the *LA1-3B* gene and that the distance between the two is less than

300 bp. To confirm this result, the PCR product and cosmid A11100 were sequenced with primers 1R and LH3. The sequence from these primers overlapped and the 1R-primed sequence read directly into the 5' region of the *LA1-3B* gene. This result mapped the distance between the two genes as less than 295 bp (see figure). Analysis of the *LA1-3B* gene 5' region, and the sequence between the putative first exons of *LA1-3B* and *BRCA1*, for potential promoter or enhancer sites revealed seven potential

'CAT' boxes as well as other motifs (see figure). No sequences likely to correspond to a TATA box were found.

The proximity of these two genes raises the possibility of shared promoter and/or enhancer sequences and thus coregulation of expression. There are a number of examples of such bidirectional promoters, including those directing the expression of the $\alpha 1$ and $\alpha 2$ collagen genes⁵, of the dihydrofolate reductase gene and the human homologue of the bacterial *MutS* gene⁶, of *Surf-1* and *Surf-2* (Ref. 7) and the Wilms' tumour *WT1* and *Wit-1* genes^{8,9}. Recent work on the collagen genes provides clear evidence for a bidirectional promoter involving non-TATA elements both between and within the exons of both genes, all of which are critical for the optimal and coordinated expression of both genes⁵. It is therefore conceivable that the 'CAT' boxes and other motifs found between the *LA1-3B* and *BRCA1* genes are important for the expression of both *LA1-3B* and *BRCA1*.

A model of dis-coordinate expression of *BRCA1/LA1-3B* would predict a decrease of *BRCA1* expression with the increased expression of *LA1-3B*, as is seen in ovarian cancers. Alternatively, *BRCA1* expression could be quenched by antisense RNA were an additional 5' exon to lie embedded within the *LA1-3B* gene. Both mechanisms would lead to the effective downregulation of *BRCA1*, and would remain consistent with a tumour-suppressor model for this gene.

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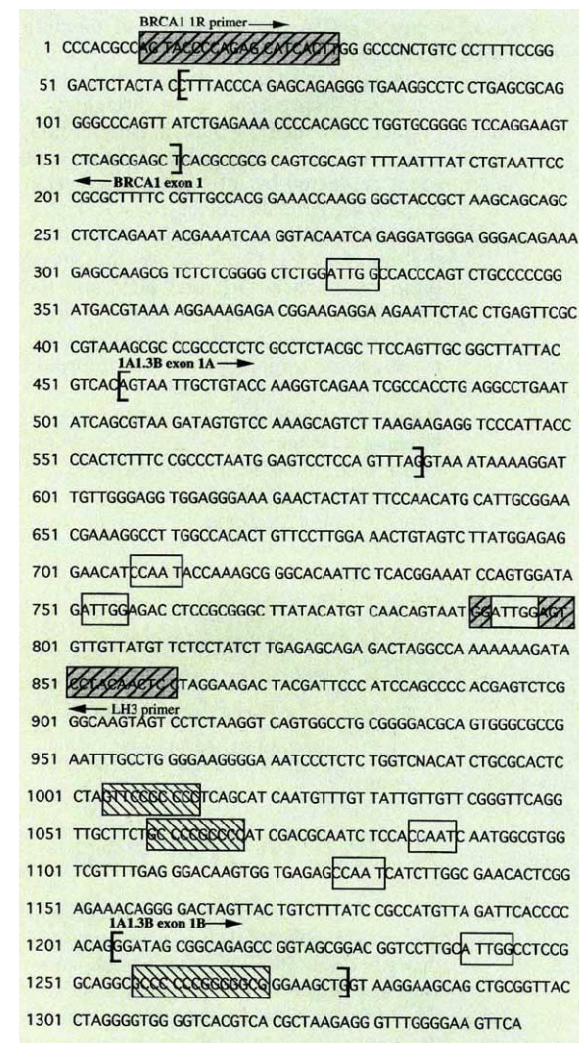
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Sequence analysis of the region between the *BRCA1* and *LA1-3B* genes. Brackets, exon sequences; hatched boxes , positions of primers *BRCA1* 1R and LH3; open and hatched boxes , positions of CAT and GC boxes, respectively.

of coregulation of these two genes by a bidirectional promoter and the potential involvement of *LA1-3B* in breast and ovarian tumorigenesis.

During the search for *BRCA1*, we isolated a number of candidate genes^{3,4}. One of these, *LA1-3B*, was isolated from a complementary DNA expression library with antisera to the ovarian cancer marker CA125, and was characterized in our laboratories². *LA1-3B* maps to the same P1 artificial chromosome (PAC) clone as

result, the PCR product and cosmid A11100 were sequenced with primers 1R and LH3. The sequence from these primers overlapped and the 1R-primed sequence read directly into the 5' region of the *LA1-3B* gene. This result mapped the distance between the two genes as less than 295 bp (see figure). Analysis of the *LA1-3B* gene 5' region, and the sequence between the putative first exons of *LA1-3B* and *BRCA1*, for potential promoter or enhancer sites revealed seven potential

Mind control

SIR — The mania for research publication that is sweeping the world, POPS (publish or perish syndrome), is an example of a disease caused by a "mind virus"¹. The vector is the scientific journal, which carries the virus in the form of communications world-wide to susceptible victims. Fear of the wrath of the employing authority, together with the promise of financial reward or better job prospects for published articles, potentiates the 'virus' and causes the number of articles and journals to proliferate, in turn infecting a new population of susceptible hosts. The external source of energy required for the propagation of the infection is the research grant.

The infectivity of the virus is not very high, as there are various 'immune' control mechanisms. One of these is the journal referee but, judging from recent reports, this mechanism is not very effective^{2,3}. Those 'viruses' which escape this mechanism and end up being published still do not have a high infectivity rate, as a large percentage are never again quoted⁴. A second control

mechanism is the inherent resistance to research work in many individuals. This is often broken down by pressure from the employing organization, but a considerable number of individuals have over the years built up a strong immunity to this pressure and successfully escape infection.

Factors enhancing resistance to the 'research virus' are of great value as the vectors are already saturated; even the multiplication of these vectors in recent years cannot cope with the virus load. I think a very good case could be made for more vigorous control of the 'research virus'. Perhaps we should start by limiting the external source of energy, or possibly the vectors.

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Visceral leishmaniasis and AIDS

SIR — Visceral leishmaniasis (VL), a protozoan infection transmitted by sandflies, has a world-wide distribution that includes major endemic regions in the Mediterranean basin, East Africa, the Middle East, the Indian subcontinent and South America. More than 500,000 clinical cases occur annually (P. Desjeux, WHO; personal communication) and more than 200 million people are exposed to infection¹. Skin tests and serology indicate that most individuals who receive an infective sandfly bite resist progression to clinical VL and that the ratio of asymptomatic to clinical cases ranges between 5 to 1 (Kenya), 10 to 1 (India) and 18 to 1 (Brazil)². VL is primarily rural and suburban, but there is a trend towards urbanization (which has recently affected several state capitals in northeastern Brazil³), whereas HIV primarily becomes established in urban foci, from which it is disseminated into suburban and rural localities. Both VL and HIV predominate in males, although VL is often acquired at an early age as infant kala azar.

In the Mediterranean region, the recent shift in the age group prevalence of VL from infants to adults is not due to oscillations in VL incidence but is seen only where HIV is present and is in part associated with activation of disease in hitherto undetected asymptomatic carriers who are also infected with HIV. Thus, based on the small samples available from this area of low endemicity, most recent

VL cases have been in HIV patients, with several-fold increases recorded in the rate of VL associated with HIV⁴ (in southern Europe 50 % of VL adult cases are now related to HIV infection), in parallel with the steadily rising prevalence of AIDS: 3–7 % of HIV patients in this region develop VL and incidence has in some areas doubled in the past 2 years. Circumstantial evidence suggests that VL can also be transmitted among drug users by syringe passage.

VL/HIV co-infections present special problems. Indirect methods of diagnosis, such as serology, frequently fail; direct methods are reliable but have less value in treated and relapsing patients, are invasive and require skilled microscopy. Treatment of VL with conventional drugs, such as antimonials, amphotericin B, pentamidine and allopurinol, gives poor results with HIV patients; more than 40% relapse or have persistent chronic infections, demonstrating the importance of the immune response during chemotherapy. Studies are required to select better drug combinations for treating VL in the immunocompromised or for providing long-term prophylaxis, and to determine whether new DNA-based detection methods provide a solution to the diagnosis of persistent infections. Release of patients prone to relapsing back into VL-endemic areas may sustain or generate new outbreaks.

Few VL/HIV co-infections have been

reported from Central and South America, but the epidemiological features of both infections are similar to those of Europe. In the Americas, at least 1.5 million people are thought to be infected with VL, with 80,000 acquiring infection each year: both VL and HIV prevalences are rising and the rate of increase in HIV infection is much higher than in Europe. Further, HIV is spreading to suburban and rural areas, while VL is becoming urbanized. It seems inevitable, therefore, that VL/HIV co-infections will become commonplace in the Americas. A similar outcome may occur in India, where HIV infection is spreading, even though rural populations such as that of Bihar State, where VL has been epidemic, at present seem protected by infrequent interaction with HIV carriers in the cities⁵.

Overall, these comparisons indicate the need to be alert to the increasing sympatry between VL and HIV and the predictable rise in VL/HIV co-infections, and the need for a sense of urgency to the more effective management of outbreaks of VL.

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A line in the sea

SIR — A line in the sea (J. A. Yoder *et al.* *Nature* **371**, 689; 1994) has been previously described in 1960 by the french sailor Bernard Moitessier in his book *Vagabond des Mers du Sud*. In this account of his voyage in the South Atlantic between the Fernando Islands and the West Indies, about 150 miles southeast of Trinidad, he writes of: "a perfectly straight frothy line (in the sea), like a taut string, . . . vanishing in the distance" (*une ligne écumante bien droite, comme tracée au cordeau, . . . se perdant dans le lointain*). He also observed a rapid change in sea colour (from green to blue), which he interpreted as a change in temperature.

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Mortgaging the Earth

Scott Barrett

Faith and Credit: The World Bank's Secular Empire. By Susan George and Fabrizio Sabelli. Penguin: 1994. Pp. 288. £6.99 (pbk).

I MET recently a developing country's former ambassador to the United Nations. He played a leading role in arguing for a new international economic order in the 1970s, one that would provide a measure of economic justice in the relations between North and South. "Back then," he told me, "we thought we could change the world." After a moment's hesitation, he added: "What we found is that the world has changed us."

This book chronicles that change, and its authors agree with the ambassador's assessment that the change has been for the worse. If anything, North-South economic divisions have widened over the past two decades. To George and Sabelli, the blame lies with a single organization: the World Bank. They argue their case well. Anyone new to this area will put the book down not only convinced that the World Bank has made a botch of its job, but also angry that the institution has been blind to its errors and that no member government has intervened to change the bank in the light of the evidence of its repeated failures. Yet the case made by the authors reads like the prosecuting attorney's opening remarks at a trial. The novice juror may be convinced, but he or she has heard only one interpretation of a selection of the relevant facts. The experienced juror will know that the defence, if given a chance, will present additional evidence and offer a different interpretation. The defence has no voice in this book, however.

To be fair, I'm not sure that this is a criticism. The World Bank has defended its own case often enough, and if this book can't find any virtue in what the bank has done, then the bank's own publications often fail to find any fault. And the interpretations offered by both are similarly suspect. In *Faith and Credit*, the bank gets the blame for a vast number of woes, while the bank, in several of its own publications, credits itself with a quantity of successes. My sense is that its record is more mixed, and that it hasn't been as influential — for better or for worse — as either George and Sabelli or the bank itself would have us believe.

The bank was created in 1944 to promote both postwar economic reconstruction and economic development. Over the years, priority in development has been given to poverty reduction, and more recently the bank has assumed the objective of promoting sustainable development. The bank advances these

aims by lending money to countries at rates that are lower than these countries could obtain on their own. Increasingly, sectoral loans are conditional on certain policy changes that encourage the opening up of markets. For example, an agricultural sector loan may require that subsidies for fertilizers be reduced. These loans are called structural adjustment loans, and it is these that most irk George and Sabelli.

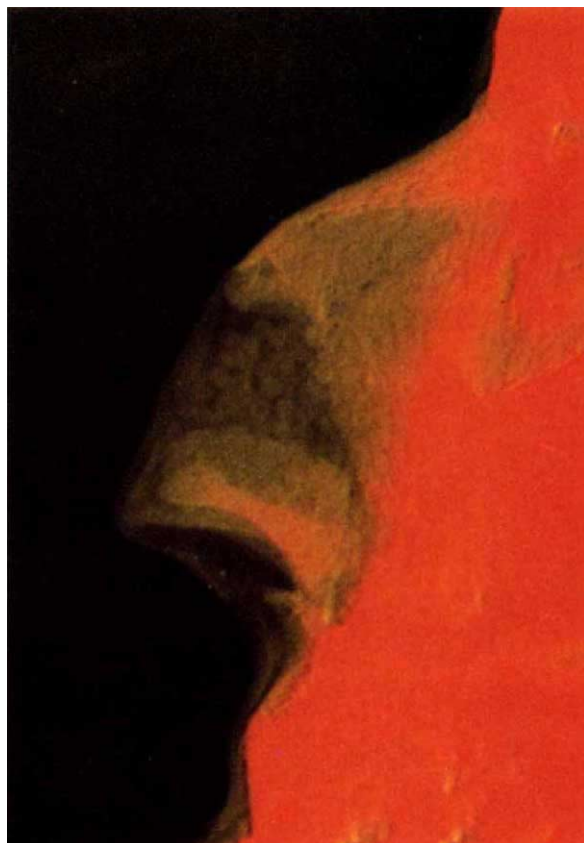
The authors argue that these loans have been a disaster, and they cite numerous examples. Although the authors do not say so, however, one can also find successes. But what are we to make of these observations? Neither, on its own, is sufficient to tell us whether structural adjustment has failed or succeeded. To know that, one would need to know how all these economies would have performed without structural adjustment, and by definition that is something which we can't observe (although it might be inferred by a careful econometric analysis). Although the authors argue that economies undertaking structural adjustment have not per-

formed well, and that their environments have been degraded in the process, what they do not document is whether these economies and environments would have fared better or even worse without structural adjustment.

More importantly, *Faith and Credit* does not offer any alternative to the bank's own policies. The closest that the authors get to proposing an alternative is in praising the views of a former World Bank economist, Herman Daly. His views are certainly different from the bank's orthodoxy — its faith — but that doesn't make them better. The bank has been a champion of trade liberalization, whereas Daly believes that countries should seek self-sufficiency. But you and I are not self-sufficient. Why should we believe that countries would be better off if only they didn't trade? Similarly, the view expressed in the book that the bank should assist in financing strategic industrial and trade policies is at best high in risk and at worst pure folly.

Related to this, one might ask why the bank should receive all the blame for the failures of development. Why shouldn't the other development banks and institutions, the governments of developing countries themselves and academic economists share the blame? The book argues that the regional and bilateral development banks have uncritically embraced the World Bank's own policies, that the governments of developing countries have

FORGET reindeers — this is an X-ray image of Pharaoh Ramesses II's nose. The mass of dark specks are grains of peppers, used as a preservative by the embalmers. The bone-like object above the nostril is in fact a plug of solidified resin designed to hold the pepper in place. The picture is reproduced from *Mummies: A Journey Through Eternity* by Françoise Dunand and Roger Lichtenberg, a compact and ingeniously illustrated guide to these Egyptian antiquities. Thames and Hudson, £6.95 (pbk).



had little option but to do the same and that academics are guilty of either blindly accepting the same orthodoxy as the bank or of being too timid to voice any opposition, not least because that might mean being struck off the bank's list of consultants. So, according to George and Sabelli, the bank is to blame not only for its own actions but also for everyone else's. This claim is absurd.

The record of development is indeed disappointing, but ditching either the World Bank or economic theory won't enrich the lives of the destitute in poor countries. The problems of development are complex. Their solutions are complex too. If you are interested in understanding them, then you should read Partha Dasgupta's *An Inquiry into Well-Being and Destitution* (Oxford University Press, 1993). I would certainly recommend *Faith and Credit* to anyone thinking of joining the World Bank — one should acquire a critical attitude toward any institution. But I would much rather that they read Dasgupta's book. In it they will not find a litany of complaints about the World Bank. They will find instead something precious: a deep understanding of the problems of the destitute in poor countries and the means for improving their lot. □

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Science in fiction

Bernard Knight

The Bourbaki Gambit. By Carl Djerassi. University of Georgia Press: 1994. Pp. 230. \$19.95.

THE cover of *The Bourbaki Gambit* carries laudatory remarks by such literary giants as Iris Murdoch, David Lodge and Arthur C. Clarke, yet after carefully reading every word of the several hundred pages, I failed to find reasons to be similarly effusive. The scientific pedigree of the author is impeccable: Djerassi is a professor of chemistry at Stanford University with an impressive list of medals and awards for chemical prowess, including the first synthesis of a steroid oral contraceptive, which earned him the title, quoted in the blurb, as 'Father of the Pill'. He has published prose and poetry, including a previous novel *Cantor's Dilemma*, which he describes in his three-page foreword as being the first in a tetralogy, of which *The Bourbaki Gambit* continues "to shine the unforgivingly bright light of contemporary life on today's scientists".

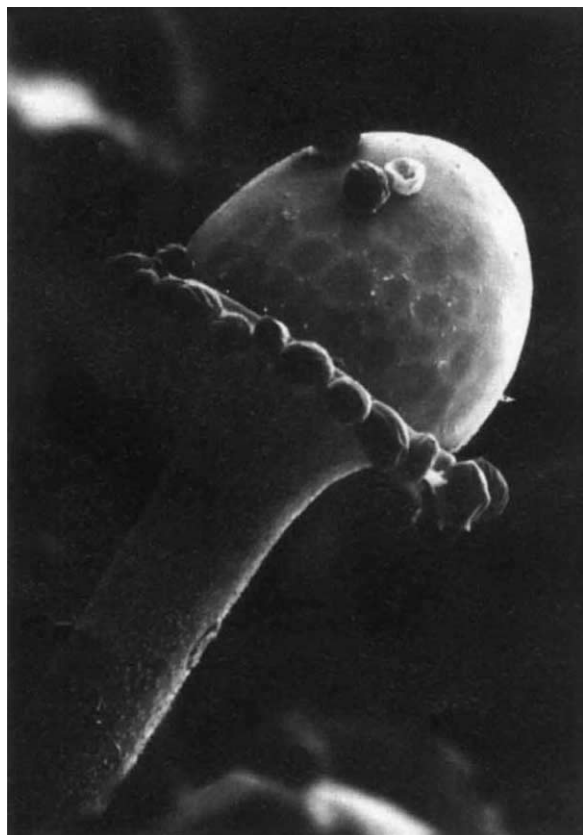
The thrust of this pretty straightforward yarn is that four geriatric scientists from various parts of the world become increasingly disgruntled with the downgrading of their research funds or their enforced retirement, especially when their name-

plates are unscrewed from their office doors on the day they leave. As a collective act of revenge, to show the world of academia that superannuation is not synonymous with creative paralysis or cognitive atrophy, they decide to construct a communal research publication of Nobel proportions, concealed under a pseudonym. It is not made sufficiently clear — at least not to me — why this ploy should have been expected to rock the foundations of the scientific establishment, apart from generating some surprise that any egotistical researchers would be willing to hide the light of their creativity under a bushel of anonymity. The idea, which provides the title of the book, came from a real-life series of publications where a group of mathematicians used the fictional name of Nicholas Bourbaki.

To my mind, the story parts company with credibility because of the apparent ease with which a world-shaking piece of research is gestated with virtually no personal access to laboratory facilities by the main members of the geriatric team. Following a series of dinner parties, a week in Capri and lots of faxes, they come up with the polymerase chain reaction (PCR), which of course actually did gain the Nobel prize in 1993. Not surprisingly, the publication they choose for their jolly jape is *Nature*, but their young lady worker jumps the gun by mailing off a couple of copies prematurely. For devotees of *Nature*, there is an amusing account of how the journal expedited publication with remarkable speed: "There were no comments by the referees" she continued. "Nothing; just a note from London that the manuscripts were accepted."

Most of the laboratory work necessary to discover and develop PCR seems to have been performed *en passant* by this young postgraduate as a preliminary to her PhD. Most of Chapter 19 is devoted, by way of dialogue, to an explanation of DNA, base sequences and the principles of recombinant and amplification techniques including six diagrams — a strange sight on the pages of a novel. The writing is competent and flows easily, and the characterization is excellent, avoiding the cardboard figures that too often abound in "science in fiction", as the author himself categorizes his genre.

The problem is the storyline, which is longwinded and lacks any surprises. The plot is virtually stated at the beginning and then travels along sedately to the end of the book, where the reader is left feeling "so what?". The several feeble attempts to inject some mild and irrelevant sexual content overshadow some much more interesting side-paths, such as a rather bitter exposé of the scientific hierarchy in Japan. I am genuinely not trying to be gratuitously unkind, but I feel that what could have made an interesting short story has made a rather tedious novel.



THE fungus *Rhizopus oligosporus* has been used for centuries as a fermentation agent in the production of tempe, an oriental soybean cake. This picture is taken from *Microfungi* by S. Gravesen, J. C. Frisvad and R. A. Samson, which concentrates on the filamentous fungi (the moulds) and their role in biodeterioration and biotechnology. Sections also cover mycotoxins, allergies and fungal infections. The book concludes with a description of 34 common mould species. Published by Munksgaard, Nørre Søgade 35, Postboks 2148, 1016 Copenhagen, Denmark. Price is DK320.

According to the author's foreword, the object of the story is to emphasize the conflict between collaborative research and the intense desire of scientists for personal recognition. This hardly seems to need emphasizing: the eternal professional jealousies, the race for publishing priority and the endemic back-stabbing is universally known as the very stuff of which the scientific establishment is made. Perhaps a more engaging novel could have been written around a murder at an international congress, where passions in the lecture halls, committees and presidia not infrequently approach homicidal proportions! □

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The middle landscape

John de la Mothe

Regional Advantage: Culture and Competition in Silicon Valley and Route 128.

By AnnaLee Saxenian. Harvard University Press: 1994. Pp. 226. \$24.95, £19.95.

Technopolis: High-Technology Industry and Regional Development in Southern California. By Allen J. Scott. University of California Press: 1994. Pp. 322. \$35.

Restructuring for Innovation: The Remaking of the US Semiconductor Industry. By David P. Angel. Guilford: 1994. Pp. 216. \$27.95.

Future Imperfect: The Mixed Blessings of Technology in America. By Howard W. Segal. University of Massachusetts Press: 1994. Pp. 245. \$40, £38 (hbk); \$15.95, £15.20 (pbk).

THE 1980s was a period of painful and far-reaching adjustment in the world economy. The term 'globalization' entered popular parlance and seemed to suggest that location no longer mattered. Prophets of industrial competition predicted 'the end of geography'. Companies had begun to invest off-shore in foreign labs without actually moving there. World financial centres such as London, New York and Tokyo became the hubs for overnight currency transactions. The race for new products became a matter of highly mobile intellectual resources rather than highly fixed natural resources. Quality mattered, not quantity. Value added was no longer based on what one had, but on what one did. Mass production gave way to flexible manufacturing. Influential economists began to talk of a transition from an economy based on objects to one



American Progress by John Gast (Gene Autry Western Heritage Museum). The picture is reproduced from the cover of *Does Technology Drive History?* edited by Merritt Roe Smith and Leo Marx. The 12 contributors, most of them historians, explore the enduring question of to what extent, and by what means, does a society's technology determine its political, social, economic and cultural forms. It is a debate that was launched by Karl Marx with his provocative remark that "the hand-mill gives you society with the feudal lord; the steam-mill, society with the industrial capitalist". MIT Press, \$35, £31.50 (hbk), \$16.95, £14.95 (pbk).

based on ideas. Policy analysts began questioning whether or not the nation state actually mattered any longer. Indeed, with an economy driven by intangible investment, foreign direct investment and innovation, the meaningful factors of production could no longer be controlled by governments. The world indeed seemed a changed place. And perhaps nowhere was this more starkly felt than in the United States.

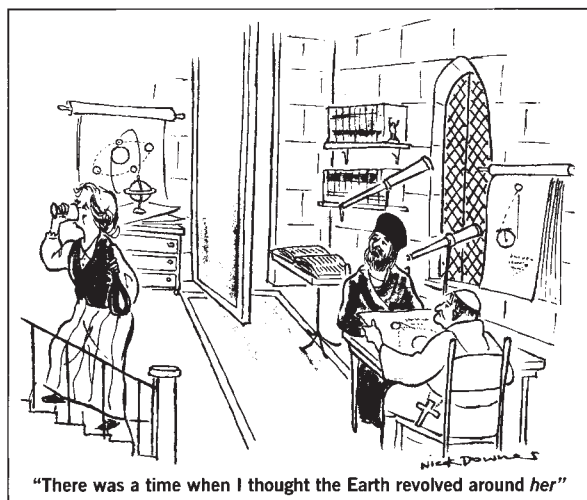
After three decades of technological and commercial domination, those industries that had come to represent US strength, such as aerospace, electronics and space equipment, began, in the 1980s, to lose both market share and technological leadership, principally to Japan. By 1986, Japan had surpassed the United States as the largest producer of semiconductors in the world, and Japanese companies were assuming an increasingly dominant position in key enabling technologies. By 1989, Japan actually ran a trade surplus of \$1.5 billion in semiconductors with the United States, while it began spending more on research and development than it did on factories and equipment. Industrial observers around the world warned that US companies could be permanently driven from the profitable segments of the high-technology markets such as computers and communication.

An almost allergic reaction ensued.

Polemic fuelled a heated, and not always constructive or clear-minded, policy debate and led influential policy analysts such as Laura Tyson, Robert Reich and Lester Thurow to ask "who's bashing whom?", "who is us?" and "who will own the twenty-first century?". Less profound thinkers gave themselves up to finger pointing, crass nationalism, chest beating and flag waving, all of which became something of a daily activity. Meanwhile jobs were lost, industries rusted, long-standing communities dispersed and the United States — finally — seemed truly to have lost its way. Or had it?

What is clear is that we are working within a new economic context. Unfortunately for policy-makers, however, they do not yet have a satisfactory interpretative scheme of this new world. Dominant policy frameworks continue to give privilege to commodities rather than to knowledge. But not only have we learned that competitive advantage for companies, nations and regions alike depends on intangibles such as technical know-how, specialized skills, smart infrastructure, product and service quality, and research and development; it also depends on clustering. Highly dispersed national systems of innovation cannot be depended on to deliver the kinds of adjustments and restructurings that have become essential in recent years.

It is for this reason that we can see that



More than 150 of Nick Downes's popular cartoons on science are collected together in *Whatever Happened to "Eureka"?*, published in paperback by Rutgers University Press at \$10.95.

growth, in a number of nations ranging from Italy and Canada to the United States, has been driven over the past 30 years by local systems of innovation. The so-called 'Four Motors of Europe' represent one much discussed example of clustering in which highly specialized skills (focused on software, automobiles and telecommunications) have combined in one location with locally developed infrastructures, well managed organizations, specialized financing arrangements and international investment. Silicon Valley in California and Route 128 in Massachusetts are two US examples. So if one were interested not in polemic but in the actual processes of adjustment, competition and restructuring and the factors that might account for success and failure, then one could do worse than peruse the volumes under review here.

In *Regional Advantage* by AnnaLee Saxenian, a comparative tale is engagingly told of Silicon Valley and Route 128. These are two of the leading US centres of electronics innovation and entrepreneurship. The regions are similar in many respects: both trace their origins in university research and military spending, and both faced severe downturns in the early 1980s. Today, however, Silicon Valley is flourishing once again whereas Route 128 continues in its decline.

Saxenian asks important and stimulating questions. What accounts for this difference in fortune? What was it that allowed Silicon Valley to adapt successfully to intensifying international competition, while Route 128 ceded its comparative advantage in computer design and manufacturing to the west coast? Using evidence gathered in hundreds of interviews with managers, entrepreneurs and policy-makers, Saxenian argues that despite similar histories and technologies, Silicon Valley developed a decentralized industrial system

that encourages experimentation, collaboration and collective learning among networks of specialized companies, whereas Route 128 came to be dominated by a few self-sufficient corporations.

Where Saxenian succeeds through the use of cultural history and detailed interview, Allen J. Scott's *Technopolis* succeeds by offering a highly sophisticated, if drier, empirical-cum-statistical treatment of industrial structure and spatial organization in the manufacturing system of southern California. After beginning with a brief survey of southern California's econ-

omy, he reviews in detail the growth of the region from the 1920s to the 1980s. Focusing on three major industries that have in some ways become synonymous with southern California — aircraft, electronics and military equipment — he carefully excavates the issues related to labour markets, industrial organization and the importance of local infrastructure.

Taking a slightly different — but no less fascinating — approach, David P. Angel's *Restructuring for Innovation* concentrates less on regional differences and sources of advantage and instead tells an important tale of the restructuring of the semiconductor industry. Beginning with a critical examination of the strengths and weaknesses of the US industry as it developed in the 1970s, Angel describes the competitive difficulties that US companies experienced during the past decade. In sharp contrast to other studies that have explained the loss of market share in terms of 'unfair trading practices' by Japan, this book concentrates on the manufacturing weaknesses that have developed in US practice. The recent recovery, if it is not too early to call it that, of the US semiconductor business is based on new manufacturing strategies that have finally overcome the age-old (false) dichotomy between innovation and production.

All these books point out ways in which the United States can compete. And in *Future Imperfect*, an evocative series of essays by the historian Howard Segal, we are reminded, in a lyrical way, that the preceding three books all deal in one way or another with that essential tension that has always existed in the United States between technological optimism and pessimism. One sees the repeated failure of technology to fulfil its utopian promise which, in the 1980s, created a widespread disillusionment with the American ideal of progress. The other sees technology as

the hallmark of the future. In between, technology and competition in the United States is ultimately concerned with social improvement and in this way portrays the processes of adjustment that are analysed by Angel, Saxenian and Scott — not as the end of geography but as a "middle landscape" (to allude to the title of one of Segal's most thoughtful essays) that is always full of mixed blessings. □

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New in paperback

Physics and Metaphysics: Theories of Space and Time by Jennifer Trusted.

Routledge, £9.99. An examination of the relationship between metaphysical beliefs and scientific enquiry into the nature of the cosmos — in particular into the nature of space and time. The author looks at the history of science from the eleventh century to the present to illustrate the way in which religious and mystical beliefs, as well as philosophical speculation, have motivated physicists and inspired their scientific quest. It is pitched at a level suitable for beginning students of the philosophy and history of science.

The Threat at Home: Confronting the Toxic Legacy of the US Military by Seth Shulman. Beacon, \$15. The author, a science journalist, offers a thorough and reasoned analysis of a serious environmental hazard.

Oscillations, Waves, and Chaos in Chemical Kinetics by Stephen K. Scott. Oxford University Press, £4.99. A short, accessible and well organized account in the Oxford Chemistry Primer series aimed at undergraduates. The author shows how the phenomena of oscillations, travelling waves and chaos arise in reacting chemical systems, and relates them to processes in biology, including the development of cardiac arrhythmias, nerve signal transmission and animal coat patterning.

Australian Rainforest by Paul Adam. Oxford University Press, £20, \$35. A comprehensive review of Australian rainforest, which includes accounts of its biological and human history. It will be of interests not only to ecologists and biogeography, but also to conservationists.

The Mystery of the Quantum World by Euan Squires (2nd edn). Institute of Physics Publishing, £12.95, \$24.50. An updated version of this bestselling introduction for the general reader.

Signifying Animals edited by Roy Willis. Routledge, £16.99. This book, which arose from the 1986 World Archaeological Congress, contains 19 essays that examine what animals mean to human beings around the world. The book will appeal to social and cultural anthropologists, cognitive psychologists and archaeologists, as well as to all those with an interest in animal symbolism.

A protein kinase involved in the regulation of inflammatory cytokine biosynthesis

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Production of interleukin-1 and tumour necrosis factor from stimulated human monocytes is inhibited by a new series of pyridinyl-imidazole compounds. Using radiolabelled and radio-photoaffinity-labelled chemical probes, the target of these compounds was identified as a pair of closely related mitogen-activated protein kinase homologues, termed CSBPs. Binding of the pyridinyl-imidazole compounds inhibited CSBP kinase activity and could be directly correlated with their ability to inhibit cytokine production, suggesting that the CSBPs are critical for cytokine production.

PROINFLAMMATORY cytokines such as interleukin-1 (IL-1) and tumour necrosis factor (TNF) are secreted proteins produced by monocytes and other cell types in response to many inflammatory stimuli¹. These cytokines are known to have a central role in inflammatory responses, because the administration of protein antagonists, such as IL-1ra, monoclonal antibodies to TNF- α and soluble TNF receptor, all block various acute and chronic responses in animal models of inflammatory diseases¹.

An alternative means of inhibiting inflammatory cytokine action is by decreasing production, because IL-1 and TNF are both regulated at the transcriptional and translational level²⁻⁴. A bicyclic imidazole, SK&F 86002, was found to have a noticeable inhibitory effect on lipopolysaccharide (LPS)-stimulated human monocyte IL-1 and TNF- α production (50% inhibitory concentration, IC_{50} = 1 μ M), and therefore the term CSAID (cytokine-suppressive antiinflammatory drug) was proposed to indicate this class of cytokine biosynthesis inhibitors. These agents have shown activity in a variety of animal models of acute and chronic inflammation⁵.

Further investigation indicated that the inhibition was primarily at the translational level for the following reasons. Although there was an inhibition of intracellular IL-1 β and TNF- α accumulation, there were no matching effects on messenger RNA abundance, size, protein half-life or protein secretion (ref. 6, and our unpublished data). Furthermore, poly-some sedimentation studies suggest that inhibition occurred at translational initiation (W. Prichett, personal communication).

A more direct way to ascertain the mechanism by which the bicyclic pyridin-4-yl- and related pyridinyl-imidazoles (2,4-diaryl-5-pyridin-4-yl-imidazole) act is to identify their molecular target. Using radiolabelled chemical probes for radioligand binding assays and photoaffinity labelling experiments, we report the identification, purification, complementary DNA cloning and biochemical characterization of CSBP (for CSAID binding

protein), the molecular target of the pyridinyl-imidazole cytokine inhibitors.

The drug-binding assay

The phenolic triaryl imidazole, compound 1 (Fig. 1a), was chosen as a radioligand because of its nanomolar potency in the cytokine synthesis bioassay and the relative ease of synthesis by catalytic reduction of the corresponding aryl dibromide in the presence of tritium gas. The human monocytic cell line, THP.1, was selected for these studies because of its responsiveness, after stimulation, in producing IL-1 and TNF, as well as its sensitivity towards the pyridinyl-imidazoles; this effect was comparable to that observed with human monocytes. The uptake of radiolabelled compound 1 by THP.1 cells was rapid, specific and was localized in the cytosolic fraction (data not shown). A simple and reproducible radioligand-binding assay based on size-exclusion chromatography using THP.1 cell cytosol and radiolabelled compound 1 was developed. The bound ligand eluted in the protein-containing void volume of a G-10 Sephadex column, and the radioactivity associated with this fraction was abrogated by the presence of excess unlabelled ligand and was not observed in the absence of cytosol (Fig. 1b). The binding was specific, time-dependent and reversible (Fig. 1c). A typical binding isotherm showed saturable and competitive binding (Fig. 1d). Scatchard analysis yielded a dissociation constant (K_D) of 30–50 nM with a single binding site. The binding activity was concentration-, temperature- and pH-dependent (data not shown), and was relatively stable in the crude cytosolic fraction. The sensitivity to trypsin digestion suggested that the binding entity was a protein, and size-exclusion chromatography using fast protein liquid chromatography (FPLC) suggested a relative molecular mass of ~50,000 (M_r , 50K; data not shown).

To establish the relevance of the binding activity found in THP.1 cells to inhibition of cytokine biosynthesis, we measured the ability of a large number of structural analogues, representing a wide range of potency in inhibiting cytokine biosynthesis, for their ability to compete with radioligand binding to THP.1 cytosol. The IC_{50} s for each compound tested in the two assays displayed a correlation coefficient of 0.9 (Fig. 1e)

|| Present addresses: Bristol-Myers-Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543, USA (D.G.); Ares Advanced Technology, Randolph, Massachusetts 02368, USA (J.E.S.).

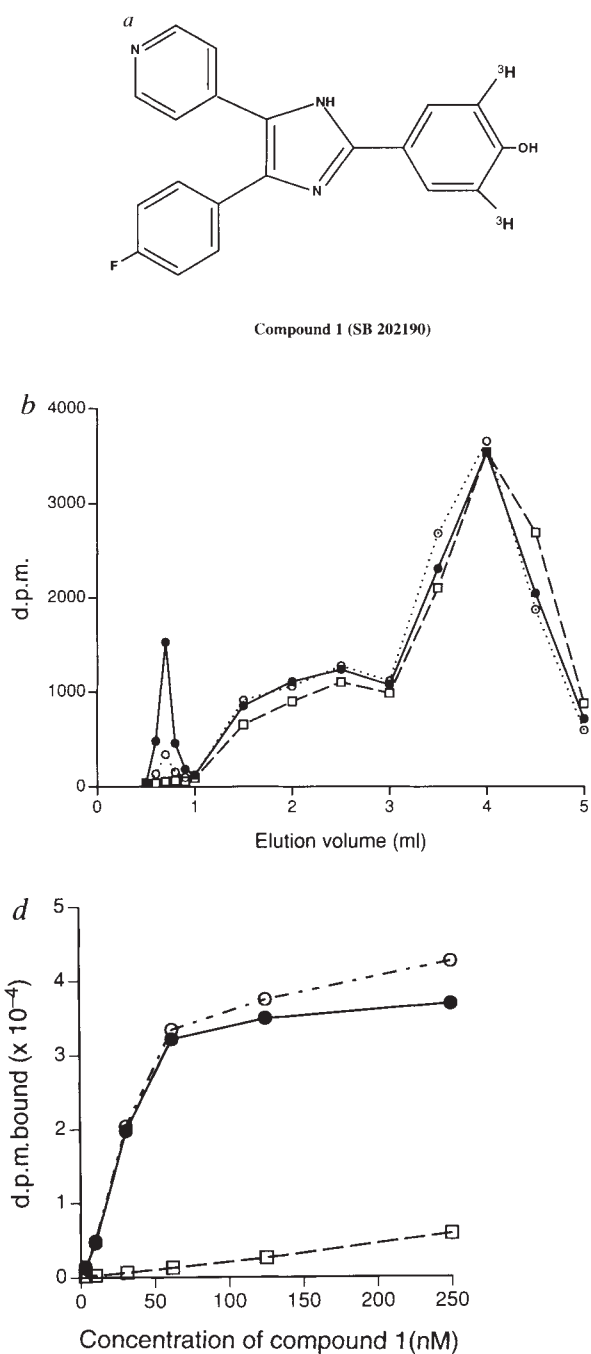


FIG. 1 Characterization of compound 1 binding in THP.1 cytosol. **a**, Structure of the radioligand, tritiated compound 1. **b**, After incubation of compound 1 with THP.1 cytosol and passage over G-10 Sephadex column, the bound and free ligand were found in the void volume (0.5 ml) and subsequent fractions (3–5 ml), respectively. $\square$ – $\square$, Radioligand alone; $\bullet$ – $\bullet$, radioligand 1 and THP.1 cytosol; $\circ$ – $\circ$, radioligand 1 and cytosol in the presence of excess unlabelled compound 1. **c**, Binding of radioligand was time-dependent ($\blacksquare$ – $\blacksquare$), specific and reversible. Excess active compounds (100-fold), unlabelled compound 1 ($\bullet$ – $\bullet$) and SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)-imidazole ($\blacktriangle$ – $\blacktriangle$), but not an inactive compound SB 202474, 4-ethyl-2-(4-methoxyphenyl)-5-(4-pyridyl)-imidazole ($\circ$ – $\circ$), added 10 min after the addition of the radioligand caused a time-dependent decrease in binding. **d**, Binding isotherm of THP.1 cytosol and radio-labelled compound 1 showing total binding ($\circ$ – $\circ$), binding in the presence of excess competitor ($\square$ – $\square$) and specific binding ($\bullet$ – $\bullet$). **e**, Scatterplot of the IC_{50} s of structural analogues in the cytokine synthesis inhibition bioassay and the radiocompetitive binding assay.

METHODS. The 100,000g supernatant of THP.1 cells (200 μg) was incubated with appropriately diluted radioligand at room temperature for 15 min and free ligand was separated from bound ligand by elution through a 1 ml G-10 Sephadex desalting column. The void volume fractions were collected and radioactivity assessed by liquid scintillation counting. For competitive binding studies, excess or appropriately diluted cold competitor was added 5 min before the addition of the radiolabelled compound. The IC_{50} s of the structural analogues in inhibiting cytokine production was determined as described previously³⁸.

with a slope of close to 1, arguing strongly that the ligand binding mediates inhibition of cytokine biosynthesis.

Identification of the drug-binding protein

The radioligand-binding assay, although useful in establishing the existence of the binding protein, was inadequate for purification of the protein. Therefore a radiophotoaffinity ligand, compound 2, was synthesized using a palladium-mediated stannylation of the aryl iodide and subsequent electrophilic radioiodination (Fig. 2a). Upon photoactivation of the THP.1 cytosol and compound 2, and subsequent analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), a single competable radiolabelled band of ~45K was detected (Fig. 2b). The M_r is similar to that previously determined by size-exclusion chromatography and the radioligand-binding assay, suggesting that CSBP is a single subunit protein. Two-dimensional protein gels further established that CSBP is a single protein with an apparent M_r of 42K and isoelectric point

of 5.2 (Fig. 2c), which was competable with a homologous non-radiolabelled ligand (not shown). Additional competition studies using various structural analogues during the photoaffinity labelling step demonstrated identical rank order potency to the binding assay (Fig. 2d).

Analysis of CSBP peptide fragments

CSBP was purified from THP.1 cytosol using a combination of the radioligand-binding assay and radiophotoaffinity labelling and subjected to trypsin and cyanogen bromide fragmentation and sequencing. The sequence of a trypsin fragment, ITAAQAL-AHAYFAQY (single-letter amino-acid code), which was not associated with radioactivity, had 85% amino-acid identity to a C-terminal sequence found in a family of serine/threonine protein kinases known as the mitogen-activated protein (MAP) kinases^{7,8}. An antiserum generated against this peptide identified a protein in two-dimensional gel western blots coincident with the previously identified radiophotoaffinity labelled protein and

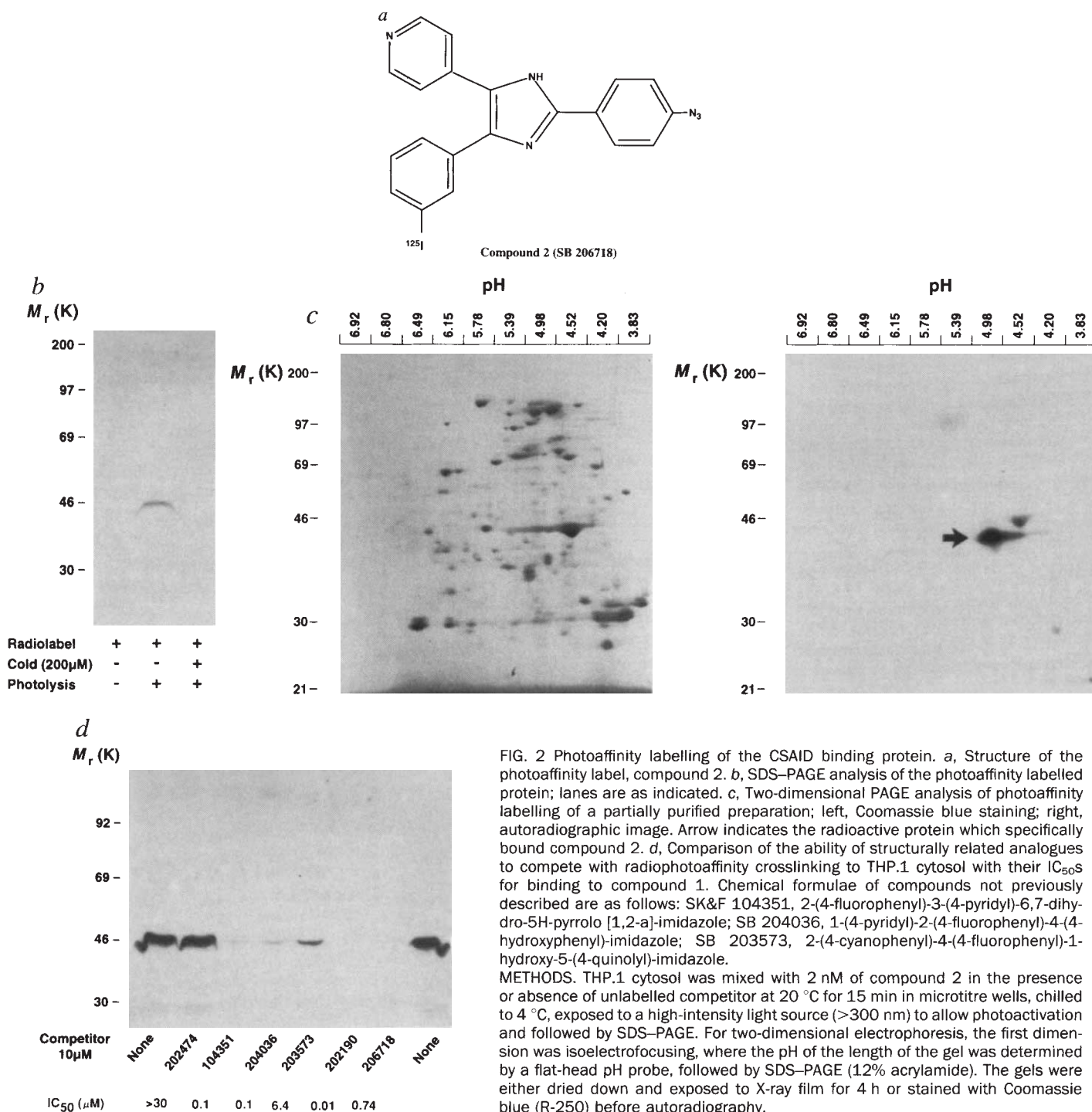


FIG. 2 Photoaffinity labelling of the CSAID binding protein. *a*, Structure of the photoaffinity label, compound 2. *b*, SDS-PAGE analysis of the photoaffinity labelled protein; lanes are as indicated. *c*, Two-dimensional PAGE analysis of photoaffinity labelling of a partially purified preparation; left, Coomassie blue staining; right, autoradiographic image. Arrow indicates the radioactive protein which specifically bound compound 2. *d*, Comparison of the ability of structurally related analogues to compete with radiophotoaffinity crosslinking to THP.1 cytosol with their IC₅₀s for binding to compound 1. Chemical formulae of compounds not previously described are as follows: SK&F 104351, 2-(4-fluorophenyl)-3-(4-pyridyl)-6,7-dihydro-5H-pyrrolo [1,2-*a*]-imidazole; SB 204036, 1-(4-pyridyl)-2-(4-fluorophenyl)-4-(4-hydroxyphenyl)-imidazole; SB 203573, 2-(4-cyanophenyl)-4-(4-fluorophenyl)-1-hydroxy-5-(4-quinolyl)-imidazole.

METHODS. THP.1 cytosol was mixed with 2 nM of compound 2 in the presence or absence of unlabelled competitor at 20 °C for 15 min in microtitre wells, chilled to 4 °C, exposed to a high-intensity light source (>300 nm) to allow photoactivation and followed by SDS-PAGE. For two-dimensional electrophoresis, the first dimension was isoelectrofocusing, where the pH of the length of the gel was determined by a flat-head pH probe, followed by SDS-PAGE (12% acrylamide). The gels were either dried down and exposed to X-ray film for 4 h or stained with Coomassie blue (R-250) before autoradiography.

distinct from the protein identified by an antiserum specific for Erk2 (data not shown). A second sequence, LNNI(V/F)KXQKLT, obtained from an 8K CNBr cleavage fragment of CSBP, was associated with radioactivity and was unique.

Cloning of CSBP cDNA

A granulocyte macrophage colony-stimulating factor (GM-CSF)-stimulated human monocyte library was screened using reverse-translated degenerate oligonucleotides based on the two peptide sequences, and a partial cDNA clone was obtained. This was used to isolate two cDNAs containing a complete open reading frame (ORF). Sequencing of the longer cDNA of 3.8 kb showed that it contained a single ORF of 360

amino acids (Fig. 3*a, b*). The second cDNA was shorter and was identical in sequence to the first cDNA except for a 75-nucleotide region within the coding region (Fig. 3*a, c*). Within this region the cDNAs share only 43% nucleotide and amino-acid identity, suggesting that they are probably products of an unusual alternative splicing event in which there is a choice of exons rather than an addition or loss of an exon. We named the products of the two cDNAs, CSBP1 and CSBP2.

Examination of northern blots indicates that the CSBPs are encoded by a mRNA of 4.2 kilobases (kb), which is about the size of the larger cDNA if one allows for polyadenylation (Fig. 4) and is expressed in all tissues examined. Southern blots at high stringency suggest that the CSBPs are encoded by a single

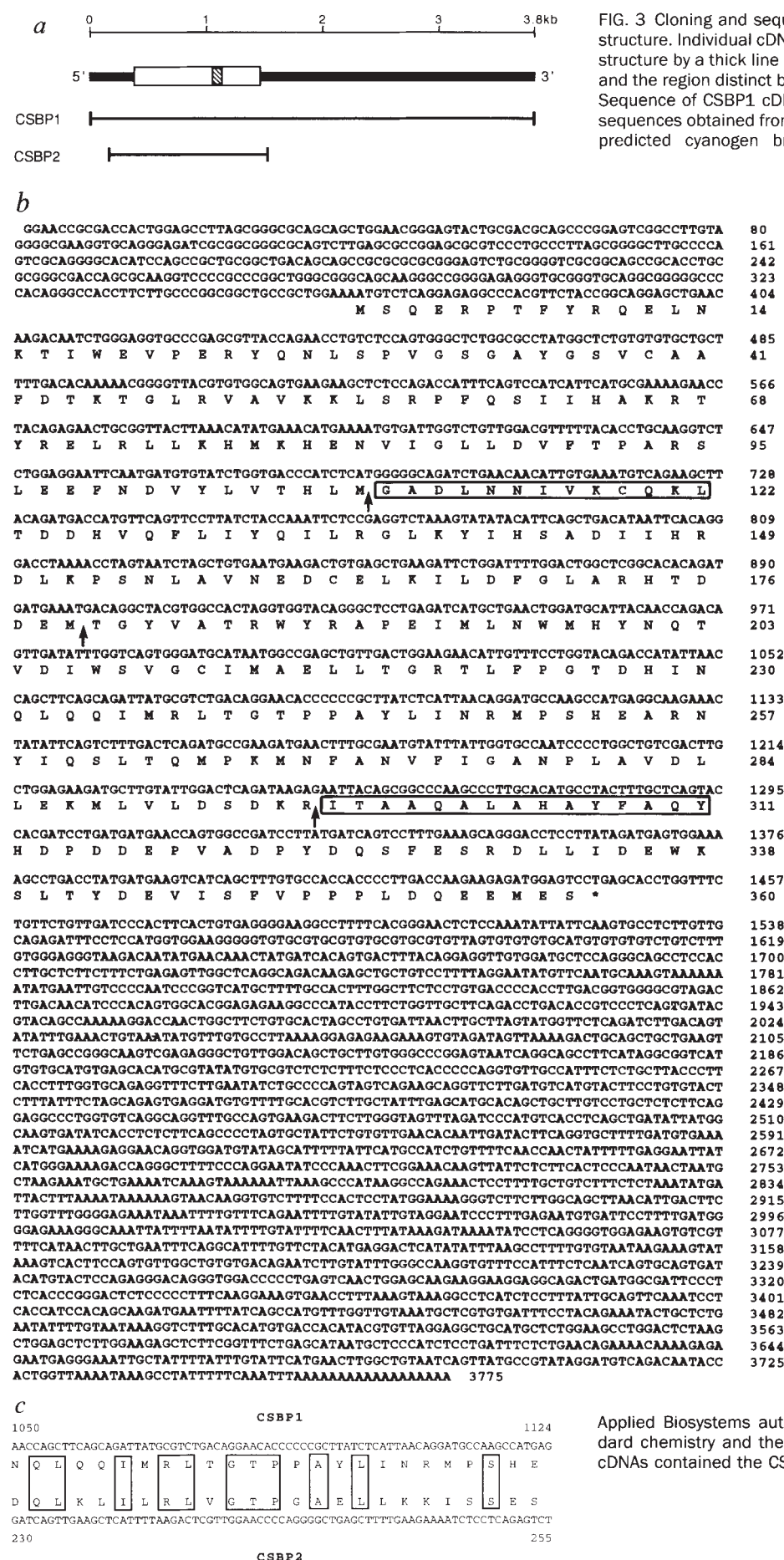


FIG. 3 Cloning and sequencing of human CSBP cDNAs. **a**, CSBP cDNA structure. Individual cDNAs are represented by thin lines and the overall structure by a thick line in which the ORF is represented by an open box and the region distinct between CSBP1 and CSBP2 by crosshatching. **b**, Sequence of CSBP1 cDNA and protein. The boxed residues match the sequences obtained from purified CSBP in THP.1 cells. The arrows mark predicted cyanogen bromide and trypsin cleavage sites for the

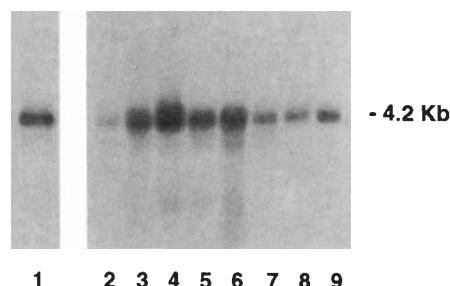
sequenced fragments. **c**, Comparison of distinct regions of CSBP1 and CSBP2 cDNA and protein sequences. The Genbank accession numbers for CSBP1 and CSBP2 are L35263 and L35264, respectively.

METHODS. From 1×10^{11} THP.1 cells suspended in 20 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 1 mM PMSF, 1 μ M pepstatin A, 1 μ M leupeptin, lysed by nitrogen cavitation, and clarified by centrifugation, CSBP was partially purified by Superose 12 chromatography in 150 mM NaCl, 10 mM sodium phosphate pH 7.0 followed by hydroxylapatite chromatography in 10 mM sodium phosphate pH 7.0, with elution through a linear gradient from 10–20 mM sodium phosphate. After concentrating using an Amicon stir cell (YM30 membrane), CSBP in the lysate was radiophotoaffinity labelled and further purified by preparative SDS-PAGE and digested with trypsin or CNBr. Trypsin fragments were fractionated by RP-HPLC on a Vydac C18 column, and individual peaks subjected to automated Edman degradation in an ABI Model 470A protein/peptide sequencer. The CNBr-digested material was fractionated by SDS-PAGE, blotted onto a Pro-blot membrane, and a single 8K labelled peptide band excised and sequenced as above. The peptide sequences obtained were used to design and synthesize two degenerate reverse-translated oligonucleotide DNA probes, which were synthesized using tables of mammalian cell codon preferences³⁹: 5'-GCYCAAGCYT-AYTTCYCYCARTA-3' and 5'-AAYAAAT-YKTSAAATTCARAA-3', where Y = C or T, R = A or G, K = G or T and S = G, C or T. Plaques (10^6) from a GM-CSF-stimulated human monocyte cDNA library in λ ZAP (Stratagene)⁴⁰ were screened with a 50:50 combination of the two degenerate oligonucleotides labelled with [γ -³²P]ATP according to standard methods⁴¹. Filters were prehybridized and hybridized at 37 °C for 24–72 h in 6 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.1% SDS and 100 μ g ml⁻¹ phenol-chloroform-extracted yeast tRNA, and washed in 6 $\times$ SSPE, 0.1% SDS at 37 °C followed by 3 M tetramethylammonium chloride solution at 37 °C for 30 min as published⁴². After further plaque purification, a partial CSBP cDNA was obtained. Its 5' sequence was used to isolate cDNAs encoding the N terminus of CSBP by hybridization to the same library as above. In total, four cDNAs were sequenced on both strands on an

Applied Biosystems automated DNA sequencer ABI 373A using standard chemistry and the sequences examined using Lasergene. Three cDNAs contained the CSBP1 sequence and one the CSBP2 sequence.

FIG. 4 Northern blot of CSBP mRNA. Lanes: 1, THP.1 mRNA; 2, pancreas; 3, kidney; 4, skeletal muscle; 5, liver; 6, lung; 7, placenta; 8, brain; 9, heart.

METHODS. Poly(A)⁺ THP.1 mRNA was prepared by guanidinium thiocyanate/CsCl-centrifugation and oligo-dT chromatography⁴¹ and 2 µg per lane electrophoresed through a 1.2% formaldehyde agarose gel and transferred to nylon using published methods⁴¹. The Multiple Tissue Northern (MTN) kit was purchased from Clontech (Palo Alto). Blots were hybridized to a 196-bp *Hind*III-*Dra*III cDNA fragment from the coding region of CSBP1, labelled with α -³²P using an oligolabelling kit (Pharmacia), with hybridization and wash conditions recommended by the manufacturer.



gene and map to human chromosome 6 (P.C.M. *et al.*, unpublished observations).

CSBP1 and CSBP2 have predicted *M_r*s of 41.5K and *pI*s of 5.45, which is very close to the size and *pI* of native CSBP estimated from two-dimensional gels (Fig. 2c). Both proteins encode the two peptide sequences obtained from the purified protein in THP.1 cells (Fig. 3b), which occur immediately after the expected cyanogen bromide and trypsin cleavage sites, respectively. The predicted cyanogen bromide fragment is 8K, matching the fragment obtained from cyanogen bromide treatment of CSBP obtained from THP.1 cells.

An initial search of genetic databases with the two CSBP sequences showed that the genes were novel but highly related to the MAP kinase family of protein serine/threonine kinases such as the mammalian extracellular regulated kinases (Erks)⁷ and the yeast HOG1 (ref. 9). After submission of this paper, the cDNA sequence of the mouse¹⁰ and *Xenopus*¹¹ homologues of CSBP2 were reported and a partial peptide sequence was reported for human¹². The homologues were discovered to be stress-responsive kinases like JNK1 (ref. 13), a more distant member of the family. Alignment of these closely related sequences (Fig. 5) shows that both CSBPs contain all 11 con-

served kinase domains and required residues, and contain consensus sequences for a serine/threonine protein kinase¹⁴. In addition, the CSBPs contain the Thr-X-Tyr motif at positions 180–182, which is known to be a regulatory site of MAP kinases⁸.

Characterization of recombinant CSBP

To confirm the identity of the cloned cDNA products with the native CSBP in THP.1 cells, and to ascertain if both putative CSBPs could bind compound 2, we expressed the two cDNAs as histidine fusion proteins in *Escherichia coli*. Both CSBP1 and CSBP2 could be specifically crosslinked to compound 2 (Fig. 6a). This crosslinking could be competed with excess cold compound with similar rank order potency to the binding assay (data not shown).

We next determined if the kinase activity of recombinant CSBP2 could be inhibited by the pyridinyl-imidazole compounds by expressing CSBP2 as a FLAG-fusion protein in a *HOG1*-deleted strain of yeast⁹. In this strain, p38 kinase has been reported to complement the *HOG1* deletion by allowing growth in high osmolarity¹⁰. Similarly, we find that CSBP2 is tyrosine-phosphorylated by the HOG1 kinase PBS2⁹ (data not shown).

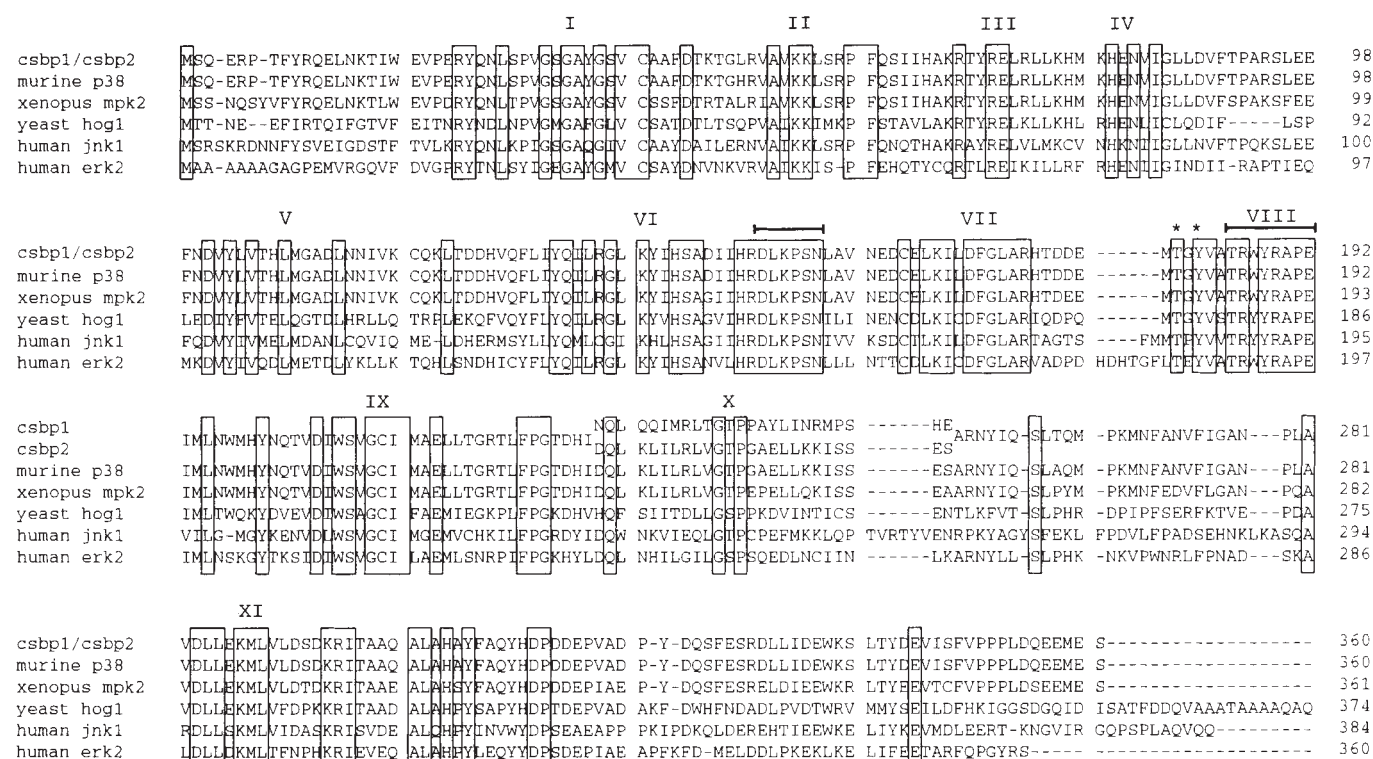


FIG. 5 Alignment of human CSBPs with members of the MAP kinase/Erk family of serine/threonine protein kinases. The closest homologues were discovered using BLAST⁴³ and the final alignments determined using GENEWORKS (Intelligenetics, Mountain View, CA). Identities are boxed. Roman numerals indicate the 11 conserved protein kinase

regions¹⁴, and the overlined residues the conserved serine/threonine kinase domains. Asterisks mark the conserved Thr and Tyr residues that are phosphorylated on activation of MAP kinases by MAPKK. The murine p38 kinase has only two differences to human CSBP2 at positions 48 (Leu to His) and 263 (Thr to Ala).

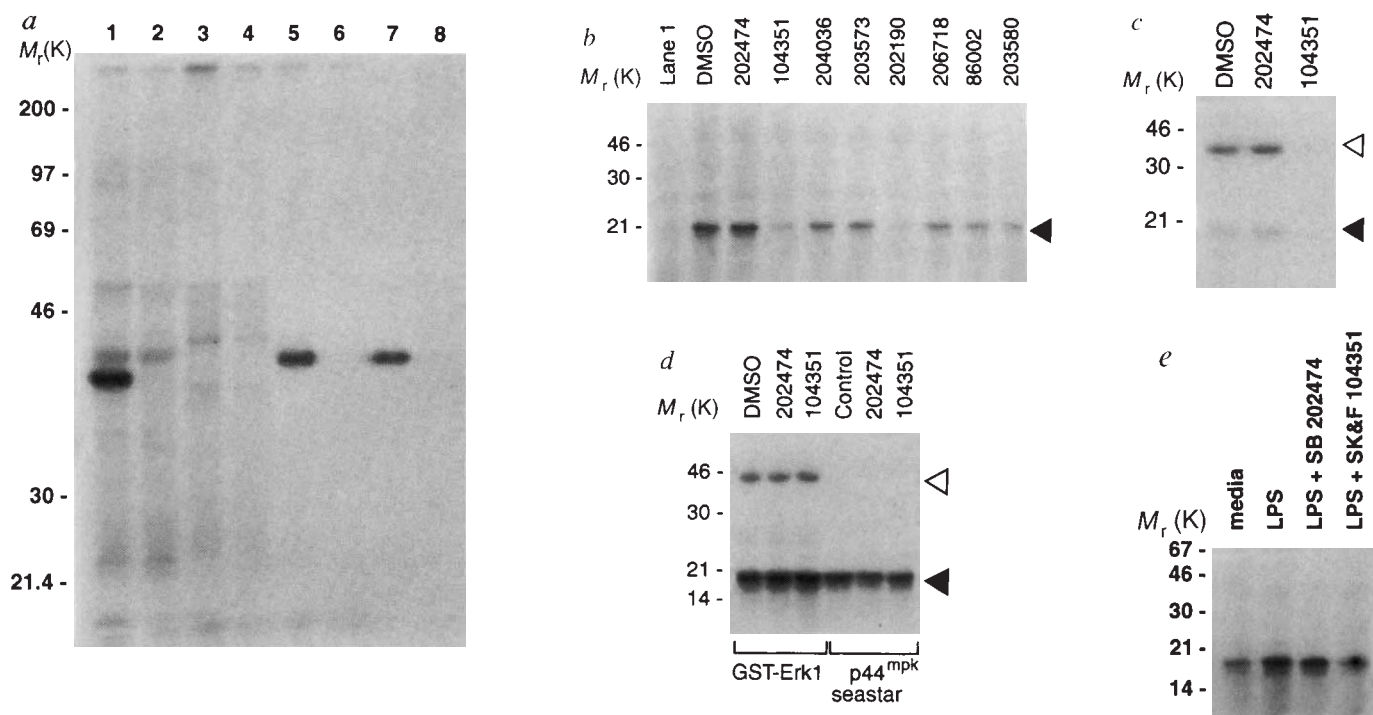


FIG. 6 Radiophotoaffinity crosslinking and kinase activity of recombinant CSBPs. **a**, Lysates of *E. coli* expressing MetAla(His)₆CSBP1 (lanes 5 and 6) or MetAla(His)₆CSBP2 (lanes 7 and 8) or control vector (lanes 3 and 4) and THP.1 cytosol (lanes 1 and 2) were subjected to photoaffinity labelling using compound 2, done as in Fig. 3, in the absence (lanes 1, 3, 5, 7) or presence of excess unlabelled competitor (lanes 2, 4, 6, 8). The recombinant CSBPs have a higher M_r than THP.1-derived material, presumably due to the N-terminal histidine fusion tag. **b**, Phosphorylation of myelin basic protein (arrowhead) by immunoprecipitated yeast-expressed FLAG-CSBP2 in the absence or presence of 5 μ M of various pyridinyl-imidazole compounds as described in Figs 1 and 2. Lane 1 is an immunoprecipitate of yeast containing control vector and is devoid of kinase activity. **c**, Phosphorylation of myelin basic protein (solid arrowhead) and autophosphorylation (open arrowhead) by purified MetAla(His)₆CSBP2 in the absence and presence of 5 μ M SB202474 (inactive compound) or 5 μ M SB104351 (active compound). **d**, Phosphorylation of myelin basic protein (solid arrowhead) and autophosphorylation (open arrowhead) by purified *E. coli* expressed human GST-Erk1 in the absence or presence of the same compounds as **c**. Also shown is the lack of effect of the compounds on phosphorylation of myelin basic protein (solid arrowhead) by natural sea star p44^{mpk}. **e**, Phosphorylation of myelin basic protein (arrowhead) by CSBP immunoprecipitated from LPS stimulated human monocytes is inhibitable by 5 μ M SK&F 104351 but not by 5 μ M SB202474.

METHODS. A MetAla(His)₆CSBP2 fusion protein vector was constructed by using the PCR primers 5'-TAGTGGATCCTACCATGGCTCATCATCATC-ATCATCTCTCAGGAGAGGCCACGTTCTAC-3' and 3'-AGTCAAGGAATAG-ATGGTTTA-5' to amplify a 450-bp N-terminal fragment from CSBP1 cDNA. The PCR product was digested with *Nco*I and *Bgl*II and was ligated into an *Nco*I-*Bgl*II-digested CSBP2-Bluescript construct which had been altered to contain a *Nco*I site at the initiator ATG. The entire coding region was cloned into pET16b (Novagen) and the protein

expressed in *E. coli* strain BL21 DE3 through IPTG induction for 3 h according to manufacturer's instructions. The MetAla(His)₆CSBP1 fusion was created by switching a unique 0.7-kb *Dra*III-*Sal*I fragment in the pET16b-CSBP2 vector which encodes the heterogeneous region with a similar fragment from CSBP1-Bluescript. For expression in yeast, CSBP2 was re-engineered to include an N-terminal FLAG epitope (Kodak, New Haven, CT) and expressed from the copper-inducible CUP1 promoter on a 2 μ plasmid (details to be described elsewhere) in the *hog1-Δ1 Saccharomyces cerevisiae* strain JBY10⁹. Cells were grown in synthetic complete minus uracil to $A_{540}=1$, induced with 150 μ M CuSO₄ for 4 h at 30 °C, pelleted, resuspended in YEPD and incubated for 2 h before treating with 0.45 M NaCl for 10 min⁹. After collecting, cells were lysed by vortexing at 4 °C with glass beads in 100 mM NaCl, 20 mM Tris pH 7.4, 1 mM MgCl₂, 1 mM PMSF, 20 mM NaF and 2 mM Na₃VO₄ and the lysate was clarified by centrifugation at 15,000g for 10 min. Aliquots of each sample were immunoprecipitated with the anti-FLAG monoclonal antibody M2 conjugated to agarose (Kodak, New Haven, CT), washed with lysis buffer and resuspended in 30 μ l of 25 mM HEPES pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂ and 5 μ M compound as indicated. After a 10-min incubation, the kinase assay was initiated by addition of 50 μ M ATP, 5 μ Ci [γ -³²P]ATP (4,500 Ci mmol⁻¹; ICN) and 4 μ g purified bovine myelin basic protein (GIBCO-BRL) in a final volume of 60 μ l, and incubation for 10 min at 30 °C. After halting the reaction by boiling, the products were analysed by SDS-PAGE and autoradiography. MetAla(His)₆CSBP2, purified by Ni-chelate chromatography (I.R.S. and S.M.F., unpublished data, and ref. 44), *E. coli* expressed human GST-Erk1 (UBI) and active p44^{mpk} purified from sea star (UBI) were assayed at 5 μ g, 5 μ g and 50 ng per reaction, respectively. Purified human monocytes were treated with or without LPS (50 ng ml⁻¹) for 15 min. CSBP kinase activity was assessed in cell lysates in the presence or absence of compounds by immunoprecipitation kinase assay using a rabbit antiserum to recombinant CSBP2.

The kinase activity of anti-FLAG immunoprecipitates was determined in the presence of various compounds using the generic MAP kinase substrate myelin basic protein (MBP) (Fig. 6b). Inhibition of yeast-expressed CSBP2 kinase activity by various pyridinyl-imidazoles followed a similar rank order potency to that observed in the binding and radiophotoaffinity assays, and in inhibition of cytokine production. An active compound in inhibiting cytokine biosynthesis (SK&F 104351) also inhibited the weaker autophosphorylation and kinase activity of CSBP2 (Fig. 6c) and CSBP1 (data not shown), expressed in *E. coli*, whereas inactive compound (SB 202474) did not. In contrast,

the active compound failed to inhibit MBP phosphorylation by human Erk1 derived in *E. coli* or sea star p44^{mpk} (Fig. 6d) or the kinase activity of JNK1, MAPKAP kinase-2, MAPKK (S.K. *et al.* and P. Cohen *et al.*, unpublished observations) and other protein kinases such as protein kinase C, calmodulin-dependent protein kinase, and cAMP-dependent protein kinase (K. Murray *et al.*, personal communication). Finally, the increase in CSBP kinase activity in LPS-stimulated human monocytes was inhibited only by the active compound (Fig. 6e), concomitant with inhibition of TNF production in the same monocyte preparation (data not shown). Hence, by both activity and binding criteria,

the CSAID cytokine inhibitors block cytokine production by selectively inhibiting the kinase activity of CSBP.

Discussion

We have discovered through radioligand binding, radio-photoaffinity labelling, cDNA cloning, expression and characterization studies that the specific targets for a series of cytokine synthesis inhibitors are a closely related pair of novel serine/threonine protein kinases homologous to the MAP kinases. The activity of the MAP kinases such as Erk2 is activated by phosphorylation on both threonine and tyrosine in a Thr-X-Tyr motif close to the substrate binding site by MAPKK in response to an extracellular stimulation. In turn, these activated kinases phosphorylate a number of substrates on serine or threonine residues in the motif (P/L)X(S/T)P (where X is any amino acid), including phospholipase A₂ (ref. 15), MAPKAP kinase-2¹⁶, transcription factors such as c-Myc¹⁷ and c-Jun¹⁸, and translational regulators such as p90^{rk} (ref. 19) which phosphorylate the ribosomal protein S6. We have shown that binding of the pyridinyl-imidazole cytokine inhibitors specifically blocks the kinase activity of CSBP, preventing the phosphorylation of its substrate(s), which then leads to inhibition of cytokine biosynthesis. Whether the binding of compounds to CSBP also blocks its activation is not yet known.

MAP kinases belonging to different phosphorylation cascades, each responding to different extracellular stimuli, have now been identified in yeast and mammalian cells²⁰. Among these are the recently identified stress response MAP kinases JNK1¹³, SAPK α ²¹ and the CSBP homologues p38¹⁰, RK¹¹ and HOG1⁹. HOG1 is required for yeast to grow in conditions of high osmolarity but is not required for normal growth, whereas JNK1 and SAPK α respond to ultraviolet irradiation and osmotic stress by phosphorylating c-Jun, and CSBP and its homologues respond to stimuli such as heat or chemical stress, high osmolarity and also LPS¹⁰ and IL-1¹². Both HOG1 and the CSBP kinases share the parallel MAPKK-like activation motif Thr-Gly-Tyr rather than Thr-Glu-Tyr found in Erk1 and Erk2 or Thr-Pro-Gly found in JNK1, suggesting activation by an alternative MAPKK, as is the case in yeast. Some functional overlap of these stress-responsive MAP kinases is suggested by the ability of both p38 and JNK1 to complement the *hog1*- Δ 1 yeast strain^{10,22}. However, specific downstream effects of these kinases and their physiological implications have not been identified. Combined with our data, these findings add further evidence for a link between cellular stress and inflammatory cytokine production^{23,24}.

A homologue of CSBP1 has not been described to date and it is not clear why there are two nearly identical CSBPs. The situation is somewhat reminiscent of Erk1 and Erk2⁷, although the latter are not splice variants but are encoded by two separate genes. Examination of the crystal structure of Erk2²⁵, which is presumed to be similar to CSBP, suggests that the region of difference between the CSBPs is close to the Thr-Gly-Tyr activation loop and the substrate binding site and may therefore be involved in differential recognition by activating MAPKKs or altered substrate preferences.

The discovery of the role of CSBP in cytokine production furthers our understanding of the importance of protein phosphorylation in the response of monocytes and macrophages to endotoxin. Previous studies have shown that a number of intracellular substrates appear to undergo tyrosine phosphorylation^{26,27}, including the MAP kinases²⁸ and the murine CSBP2 homologue, p38¹⁰, and that generic inhibition of these pathways by tyrosine kinase inhibitors blocks TNF- α synthesis and prevents LPS-induced lethal toxicity²⁹. Serine/threonine phosphorylation has also been implicated, because treatment of macrophages with phosphatase inhibitors such as okadaic acid, which effectively increases protein phosphorylation, stimulate TNF- α production³⁰. The discovery that compounds that bind to CSBP and inhibit its kinase activity also block cytokine pro-

duction further indicates that serine/threonine phosphorylation is essential for the cytokine biosynthetic pathway.

Upon monocyte activation, IL-1 β and TNF- α synthesis are stimulated at the transcriptional level, and there is some evidence that this may in part be mediated by the transcription factors NF- κ B and AP-1³. For both of these factors, phosphorylation can play a regulatory role in transcriptional activation either through indirect phosphorylation of NF- κ B-associated protein, I- κ B or direct phosphorylation of the c-Jun component of AP-1 by p38 kinase¹⁰ and JNK1²², respectively. It has also been established that TNF- α and IL-1 β can be regulated at the translational level^{2,4,31,32}. This is the first suggestion that the translation of TNF- α and IL-1 β can be regulated through an independent phosphorylation cascade that is required for the expression of these proteins. Previous studies with reporter gene constructs in which various deletions have been made and transfected into macrophage cell lines have suggested that the regulation of TNF translations is mediated through the AUUUA repeated motif in the 3'UTR of the TNF mRNA³¹. Deletion of this region leads to constitutive synthesis in macrophage and non-macrophage cell lines³³ and in transgenic animals³⁴. With similar reporter constructs, we now know that inhibition of reporter gene expression by the pyridinyl-imidazole cytokine inhibitors is mediated through the same region (J.C.L. *et al.*, unpublished data). These AUUUA regions have also been implicated in the regulation of mRNA stability, and they associate with proteins of 37–40K^{35,36}. It is unclear exactly how these proteins may regulate mRNA stability and translation, but we speculate that the association of these proteins with the AUUUA site and perhaps the 5' end of the mRNA prevents translational initiation. This could be relieved by phosphorylation through the CSBP kinase pathway, which then causes release of the AUUUA-binding protein and translational derepression of TNF- α and IL-1 β . The pyridinyl-imidazole cytokine inhibitors, by inhibiting the CSBP pathway, would prevent this translational derepression. This model is similar to the suggested role of Erk2 in upregulating translation initiation in response to insulin³⁷. Initiation factor eIF-4E is sequestered in unstimulated cells in a complex with PHAS-1, but upon phosphorylation of PHAS-1 by Erk2, it is released and can participate in initiation of translation.

The CSAID cytokine inhibitors directed toward blocking the kinase activity of CSBPs may provide new therapeutic candidates with a spectrum of activity distinct from current anti-inflammatory treatments, and should provide useful tools for dissecting the role of this novel MAP kinase pathway in signal transduction. □

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Crystal structure of the tyrosine kinase domain of the human insulin receptor

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The X-ray crystal structure of the tyrosine kinase domain of the human insulin receptor has been determined by multiwavelength anomalous diffraction phasing and refined to 2.1 Å resolution. The structure reveals the determinants of substrate preference for tyrosine rather than serine or threonine and a novel autoinhibition mechanism whereby one of the tyrosines that is autophosphorylated in response to insulin, Tyr 1,162, is bound in the active site.

INSULIN activates a number of signalling pathways that regulate cellular metabolism and growth (reviewed in ref. 1). The insulin receptor is a transmembrane glycoprotein^{2,3} and a member of the receptor tyrosine kinase family which includes, among others, the receptors for epidermal and platelet-derived growth factors (EGF and PDGF). The extracellular portion of receptors in this family contains the binding site for its particular protein ligand and the tyrosine kinase activity resides in the cytoplasmic portion. In contrast to the EGF and PDGF receptors, which are monomeric and dimerize upon ligand binding (reviewed in ref. 4), the insulin receptor is a disulphide-linked $\alpha_2\beta_2$ heterotetramer. Binding of insulin to the extracellular α -chains is thought to cause a change within the quaternary structure of the receptor that results in autophosphorylation of specific tyrosines in the cytoplasmic portion of the β -chains: two in the juxtamembrane region (~30 residues C-terminal to the transmembrane helix), three in the tyrosine kinase domain (~300 residues), and two in the C-terminal tail (~70 residues)^{5–8}. The nature of the autophosphorylation events, whether *cis* (within a β -chain) or *trans* (between β -chains), has been the subject of debate^{9–12}.

Autophosphorylated tyrosines of the EGF and PDGF receptors serve as binding sites for proteins that contain Src-homology-2 (SH2) domains¹³, whereas the phosphotyrosines of an insulin-receptor substrate, IRS-1, rather than those of the receptor itself, are the predominant targets for SH2-containing proteins¹⁴. Although the roles of the phosphotyrosines in the juxtamembrane region and the C-terminal tail have not been fully elucidated, it is well established that autophosphorylation of the three tyrosines in the kinase domain stimulates kinase activity towards exogenous substrates⁷. The tyrosine kinase activity of the insulin receptor has been shown through kinase-inactivating point mutations to be essential for insulin signal transduction^{15,16}. A number of nonsense and missense mutations

in the tyrosine kinase region of the gene encoding the insulin receptor have been identified in patients afflicted with non-insulin-dependent diabetes mellitus (NIDDM) (reviewed in ref. 17).

We have used the multiwavelength anomalous diffraction (MAD) phasing method¹⁸ to determine the crystal structure of the unphosphorylated, apo form of the tyrosine kinase domain of the insulin receptor (IRK). The structures of several protein serine/threonine kinases (PSKs) have been reported: cyclic-AMP-dependent protein kinase (cAPK)¹⁹, cyclin-dependent kinase 2 (CDK2)²⁰, mitogen-activated protein kinase (MAPK)²¹, and twitchin kinase²². The IRK structure reveals those features that are characteristic of members of the protein tyrosine kinase (PTK) family.

Structure determination

A baculovirus/insect cell expression system was used to produce a 306-residue fragment of the β -chain of the human insulin receptor which contains tyrosine kinase activity and the autophosphorylation sites Tyr 1,158, Tyr 1,162 and Tyr 1,163 (L.W., S.R.H., W.A.H. and L.E., manuscript submitted; numbering is according to ref. 3). The N and C termini of the expressed protein were chosen on the basis of proteolytic studies performed on the entire cytoplasmic portion (M_r 48K) of the β -chain. The purified protein was not tyrosine-phosphorylated as judged by western blotting with anti-phosphotyrosine antibody and autophosphorylation experiments demonstrating phosphorylation at three sites upon addition of Mg-ATP.

Attempts to solve the structure of the unphosphorylated, apo form of IRK by molecular replacement using the cAPK structure as a search model were unsuccessful. The structure was solved by the MAD phasing method using a crystal derivatized with ethylmercuric phosphate, which binds to two cysteine thiols. Synchrotron diffraction data were collected at three X-ray wavelengths near the mercury L_{III} absorption edge. The initial MAD-

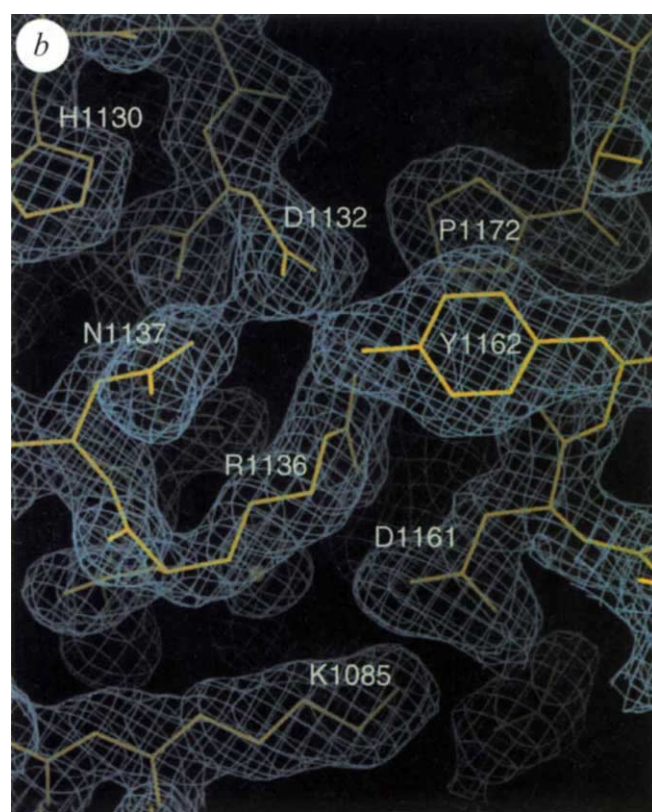
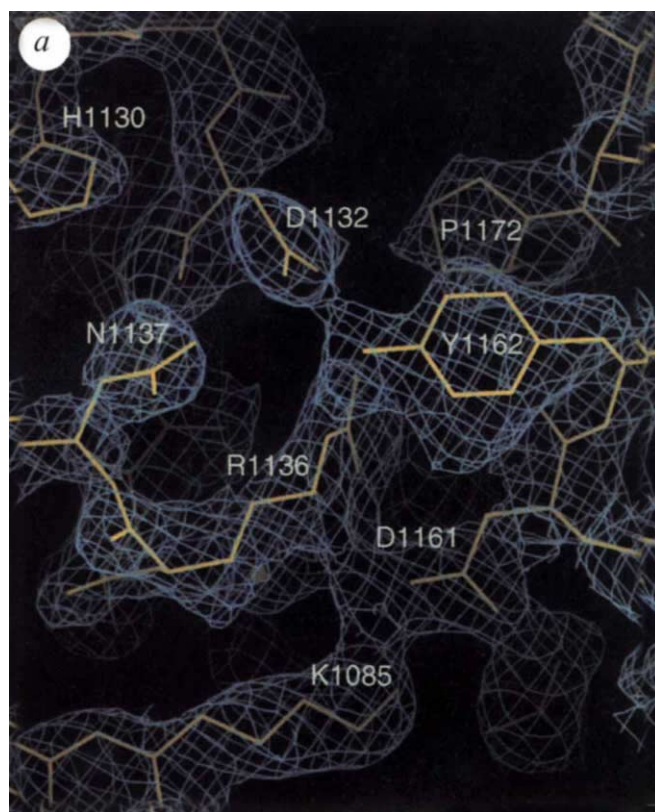


FIG. 1 *a*, The experimental, MAD-phased electron density map at 2.5 Å resolution in the region of the IRK active site, contoured at 1σ . Superposed is the refined atomic model. *b*, The corresponding $2F_o - F_c$

electron density map at 2.1 Å resolution, contoured at 1σ . Figures prepared with SETOR⁴¹.

derived phases to 2.5 Å Bragg spacings were subsequently improved through multiple cycles of model building, refinement and partial model phase combination. The structure has been refined to 2.1 Å resolution with a crystallographic *R*-value of 19.6%. The atomic model includes all but the three N-terminal residues. Electron density maps computed with the MAD-derived phases and with the phases calculated from the atomic model are shown in Fig. 1. Details of crystallization, data collection and analysis, model building and structure refinement are given in Table 1.

Overall topology

IRK is composed of two lobes with a single connection between them, similar to the kinase cores of the PSKs whose structures have been determined^{19–22} (Fig. 2*a*). The N-terminal lobe comprises a twisted β -sheet of five antiparallel β -strands ($\beta 1$ – $\beta 5$) and one α -helix (αC). The secondary structure nomenclature follows that for cAPK¹⁹; a structure-based sequence alignment is shown in Fig. 3. IRK lacks the short α -helix B which immediately precedes α -helix C in cAPK¹⁹, as do the other PSKs with known structure. The β -sheet cores of IRK and cAPK are very similar, with a root-mean-square (r.m.s.) deviation of only 0.8 Å for a superposition of 30 Ca atoms in the five β -strands. Differences in the N-terminal lobe are found in the loops between β -strands, including a five-residue insertion between $\beta 2$ and $\beta 3$ in IRK, and near the N terminus of αC (Fig. 2*c*). Although the first eight residues in the IRK structure (981–988) are relatively mobile (main-chain *B*-values >45 Å²), the backbone conformation is extended rather than α -helical; the latter was predicted for PTKs possessing the semiconserved Trp(989)-Glu sequence²³.

The larger C-terminal lobe comprises eight α -helices (αD , αE , αEF , αF – αJ) and four β -strands ($\beta 7$, $\beta 8$, $\beta 10$, $\beta 11$). The one-and-a-half-turn αEF is only a one-turn helix in cAPK and was

not identified as such in the cAPK structure report. IRK lacks β -strands 6 and 9 of cAPK, although the IRK residues that correspond to β -strand 6 follow a similar course. A superposition of 66 Ca atoms in helices αD – αH of IRK and cAPK gives an r.m.s. deviation of 1.3 Å. In the C-terminal lobe, IRK and cAPK are most dissimilar in the activation loop, between $\beta 8$ and αEF , and in the connection between αD and αE . The activation loop contains the three tyrosine autophosphorylation sites in IRK and the essential phosphorylation site Thr 197 in cAPK. In the apo IRK structure, the activation loop (five residues longer than in cAPK) contains the two short β -strands $\beta 10$ and $\beta 11$ and traverses the cleft between the N- and C-terminal lobes (Fig. 2*d*). The paths of the apo IRK and cAPK activation loops diverge at Gly 1,149 (Thr 183) and reconverge at Pro 1,172 (Thr 201). Residues 1,152–1,157 in the IRK activation loop are relatively mobile, with main-chain *B*-values exceeding 45 Å². The loop connecting αD and αE , known as the kinase insert region (~100-residue insertion in the PDGF receptor), is ten residues longer in IRK than in cAPK and contains the sequence Pro(1,099)-Gly-Arg-Pro-Pro-Pro, which is reminiscent of proline-rich sequences to which Src-homology-3 (SH3) domains bind²⁴. The three consecutive prolines in the IRK structure adopt a left-handed polyproline type II helical conformation, as do the proline-rich peptides in the SH3-peptide complexes whose structures have been determined^{25,26}. The proline-rich sequence in IRK is shorter by one helical turn than the canonical SH3-binding sequence; as yet, no IRK-SH3 interaction has been reported.

Orientation of N- and C-terminal lobes

The major difference in the global structure of IRK and cAPK is the relative orientation of the N- and C-terminal lobes. Crystallographic studies of mammalian cAPK with and without Mg-ATP and a peptide inhibitor (PKI) have revealed two conforma-

tional states for this kinase, referred to as the open and closed forms²⁷ (the closed form being active). In the open form, seen in the apo and binary (+PKI) structures, the N-terminal lobe is swung away from the C-terminal lobe by 14° and translated by 0.7 Å compared with the lobes in the closed, ternary (+PKI,

+Mg-ATP) structure. The N-terminal lobe of IRK is rotated 26° and translated 0.8 Å relative to closed-form cAPK and 19° and 2.1 Å relative to open-form cAPK. In addition to a rotational component that results in a wider cleft between the two lobes (axis perpendicular to the page in Fig. 2d), there is also a

TABLE 1 Statistics for data collection, phase determination and refinement

Data collection (20.0 to 2.5 Å)								
Wavelength (Å)	Reflections* (N)			Completeness (%)		Signal ⟨I/σ(I)⟩	R _{sym} [†] (%)	
0.9793 (remote)	22,529			95.7		22.4	2.6	
1.0061 (peak)	22,461 (37,359)‡			95.4 (93.9)		21.3 (20.7)	2.5 (2.7)	
1.0093 (edge)	22,346			94.9		20.9	2.5	
MAD structure factor ratios§								
	Observed ratios							
Wavelength (Å)	20.0 < d < 4.0 Å			4.0 < d < 2.5 Å			Scattering factors (e)	
	0.9793	1.0061	1.0093	0.9793	1.0061	1.0093	f'	f''
0.9793	0.035 (0.017)	0.019	0.034	0.037 (0.024)	0.023	0.037	−21.6	6.0
1.0061		0.054 (0.017)	0.027		0.057 (0.023)	0.032	−14.4	10.1
1.0093			0.050 (0.020)			0.057 (0.031)	−7.7	9.7
MAD phasing								
	R(^o F _T) = 0.033			⟨Δ(Δφ)⟩ = 32.8°			⟨m⟩ = 0.87	
	R(^o F _A) = 0.315			⟨σ(Δφ)⟩ = 14.6°				
Refinement¶								
Model: 303 residues, 201 water molecules, 2 EMP molecules (2,588 atoms)								
d-spacings (Å)	Reflections (N)	R-value (%)	Free R-value (%)	R.m.s. deviations				
				Bonds (Å)	Angles (°)	B-values (Å ²)		
6.0–2.1	34,747	19.6	23.2	0.011	1.6	2.2		

Expression and crystallization. A recombinant baculovirus was constructed to code for human insulin receptor residues Val 978 to Lys 1,283 (L.W., S.R.H., W.A.H. and L.E., submitted). Two amino-acid substitutions were introduced: Cys 981→Ser and Tyr 984→Phe. The expressed protein was purified from lysates of baculovirus-infected insect cells (48-h post-infection) on Q-Sepharose, Superdex-200 and Mono-Q columns. Crystals of apo IRK were grown at 21 °C by vapour diffusion in hanging drops containing equal volumes of 10 mg ml⁻¹ protein solution and the reservoir solution of 20% polyethylene glycol (PEG) 6000, 0.2 M malate-imidazole, pH 7.5. Macroseedling was required to grow crystals of sufficient size. The crystals belong to space group P2₁2₁2₁ and have unit cell dimensions of $a = 54.0$ Å, $b = 73.0$ Å, $c = 89.2$ Å when frozen. There is one IRK molecule in the asymmetric unit and the solvent content is 52%, assuming a partial specific volume of 0.73 cm³ g⁻¹. **Data collection.** All of the MAD data were obtained from one cryocooled mercury-derivative crystal with approximate dimensions of 0.6 × 0.6 × 0.07 mm. The crystal was soaked in stabilizing solution (25% PEG 6000, 0.2 M malate-imidazole, pH 7.5) which included 0.1 mM ethylmercuric phosphate (EMP) for ~40 h, after which it was transferred to stabilizing solutions that successively included 5% and then 10% glycerol. The crystal was flash-cooled in a dry nitrogen stream at -160 °C. Data were collected on Fuji imaging plates (IPs) at three X-ray wavelengths near the mercury L_{III} edge at beamline X-4A at the National Synchrotron Light Source, Brookhaven National Laboratory. The crystal was oriented such that a^* was parallel to the oscillation axis. Bijvoet pairs across the b^*c^* plane were collected on the same or adjacent IPs. Oscillation ranges of 1.7–1.9° and exposure times of 90–120 s were used. The exposed IPs were digitized with a Fuji scanner. **Data processing.** Raw IP data were converted to integrated intensities with DENZO³⁶. ROTAVATA³⁷ was used to calculate scaling parameters for each IP, and these were applied with a modified version of AGROVATA³⁷ that does not require redundant measurements. The MADSYS program package (W.A.H.) was used to extract phases and figures of merit. The experimental electron density map was computed using MAD-derived phases for reflections from 20.0 Å to 2.5 Å. **Model building and refinement.** Fitting of the polypeptide chain to the electron density was done using FRODO³⁸ on a Silicon Graphics Iris workstation. Reference was made to the cAPK structure¹⁹ during model building. COMBIN (W.A.H.) was used to combine the experimental phases with phases calculated from the partial model. Least-squares and simulated annealing refinement were done using X-PLOR³⁹, gradually extending the resolution from 2.5–2.1 Å. When the model was nearly complete, simulated annealing omit maps were computed in which ten consecutive residues were omitted from the calculation. As defined in PROCHECK⁴⁰, there are no residues in disallowed main-chain torsion angle regions and two residues, Arg 1,131 and Asp 1,156, in generously allowed regions. Side chains for the following residues were not modelled past C β owing to poor electron density: 981, 987, 1,034, 1,047, 1,096, 1,127, 1,153–1,157, 1,159 and 1,237. The average B-value for protein atoms is 23.2 Å². The side chains for Met 1,051 and Met 1,076 have been modelled in two different conformations. Water molecules whose B-values refined to >50 Å² were omitted from the subsequent round of refinement. EMP is bound to Cys 1,056 and Cys 1,234.

* Unique reflections for acentrics in point group symmetry P222 and centrics in Pmmm.

† $R_{\text{sym}} = 100 \times \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$, where I_i is the i th measurement and $\langle I \rangle$ is the weighted mean of all measurements of I .

‡ Values in parentheses are for data from 20.0–2.1 Å used for refinement.

§ Table values represent $\langle \Delta|F|^2 \rangle^{1/2} / \langle |F|^2 \rangle^{1/2}$, where $\Delta|F|$ is the absolute value of the Bijvoet difference at one wavelength (diagonal elements) or of the dispersive difference at two wavelengths (off-diagonal elements). The values in parentheses are the ratios for centric Bijvoet reflections, which would be equal to zero for perfect data and serve as an estimate of the noise in the anomalous signals. The listed scattering factors are the refined values for mercury.

|| $R = \sum_{hkl} \sum_i |F_i| - \langle |F| \rangle / \sum_{hkl} |F_i|$. $^{\circ}F_T$ is the structure factor due to normal scattering from all the atoms. $^{\circ}F_A$ is the structure factor due to normal scattering from the anomalous scatterers only, and $\Delta\phi$ is the phase difference between $^{\circ}F_T$ and $^{\circ}F_A$. $\Delta(\Delta\phi)$ is the difference between two independent determinations of $\Delta\phi$. Values given are based on calculations that did not include reflections with $m = 0$; $\langle m \rangle$ is the mean figure of merit including reflections with $m = 0$.

¶ A subset of the data (10%) was excluded from the refinement and used for the free R-value calculation until the final round of refinement, in which all of the data ($F > 2\sigma$) were used. $R\text{-value} = 100 \times \sum_{hkl} \|F_0\| - |F_c| / \sum_{hkl} \|F_0\|$. R.m.s. deviation in B-values is for bonded atoms.

rotational component along the long axis of the molecule (vertical in Fig. 2*d*, anticlockwise from above upon opening). The apo forms of MAPK and twitchin kinase also adopt open conformations with lobe rotations of 17° and 30° (refs 21 and 22, respectively), *vis-à-vis* closed-form cAPK, whereas apo CDK2 adopts a closed conformation²⁰.

The N- and C-terminal lobes of IRK are held apart by steric interactions between conserved Gly 1,005 of the glycine-rich, nucleotide-binding loop (between $\beta 1$ and $\beta 2$) and residues Phe 1,151 and Gly 1,152 of the kinase-conserved Asp-Phe-Gly sequence, near the beginning of the activation loop (Figs 2*b*, 5*b*). In contrast, the interaction of residues in αC with Asp-

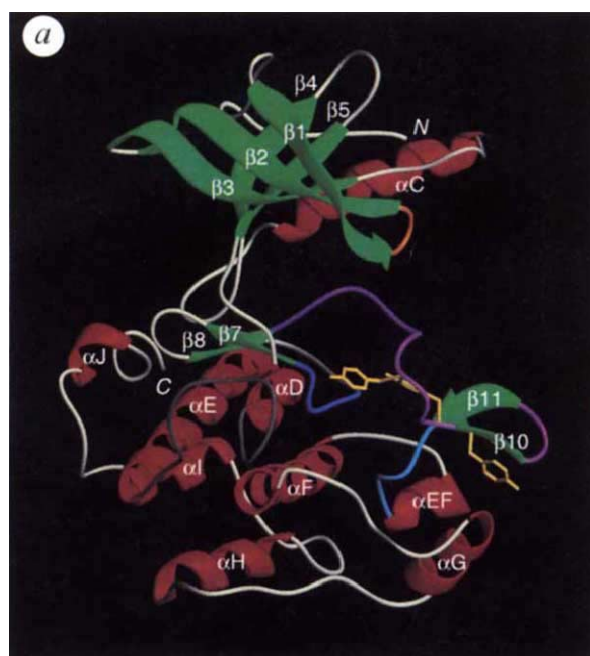
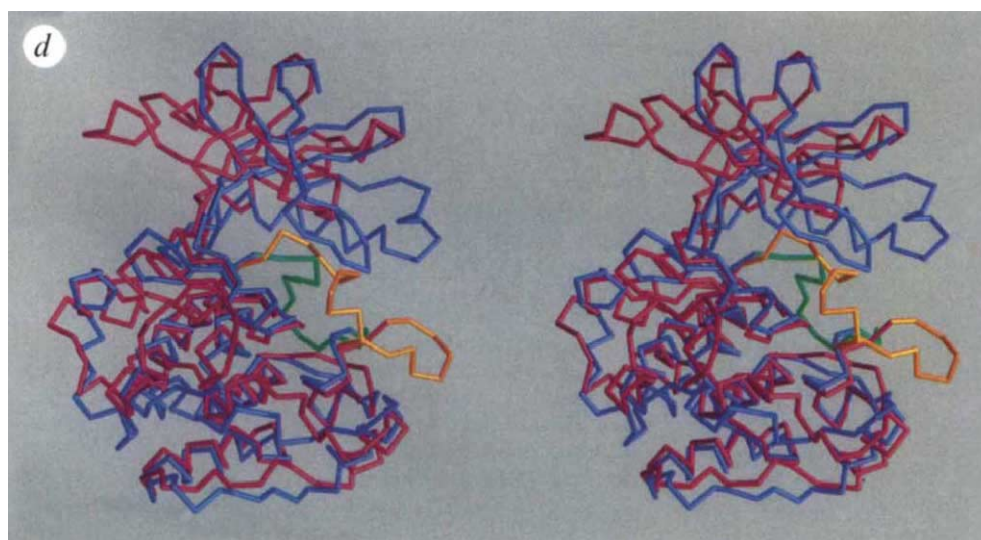
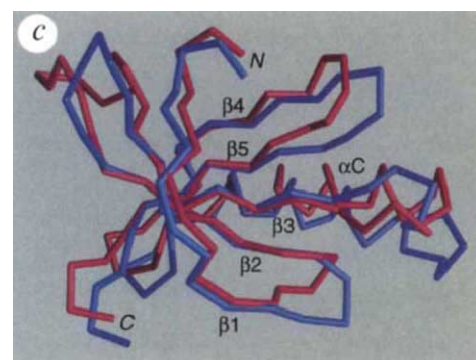
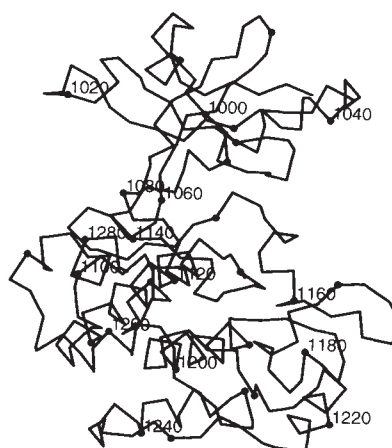
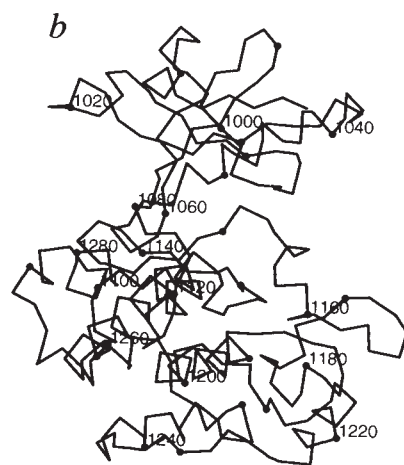


FIG. 2 *a*, Ribbon diagram of the apo IRK structure. The α -helices are shown in red, the β -strands in green, the side chains of tyrosines 1,158, 1,162 and 1,163 in yellow, the glycine-rich, nucleotide-binding loop in orange, the catalytic loop in dark blue, the activation loop in violet, the P + 1 loop in light blue, and the kinase insert region in dark grey. The termini are denoted by N and C. *b*, Stereo view of a $C\alpha$ trace of IRK in the same orientation as in *a*. Every tenth residue is marked with a filled circle and every twentieth residue is labelled. *c*, $C\alpha$ trace of the N-terminal lobes of IRK (residues 993–1,081) and cAPK (residues 40–125) in which the $C\alpha$ atoms of the β -sheet cores are superposed. IRK is in red, cAPK in blue. The view is rotated slightly from that in *a*. *d*, Stereo view of a $C\alpha$ trace of IRK (residues 981–1,283) and cAPK (residues 40–310) in which the $C\alpha$ atoms of αD – αH are superposed; colouring as in *c*. The activation loop in IRK (residues 1,149–1,170) and the corresponding residues in cAPK (183–199) are shown in orange and green, respectively. The view is the same as in *b*. Brookhaven Protein Data Bank entry 1ATP for cAPK⁴² was used in the $C\alpha$ superpositions. Preparation of figures: *a* made with RIBBONS⁴³; *b* with MOLSCRIPT⁴⁴; *c* and *d* with GRASP⁴⁵.



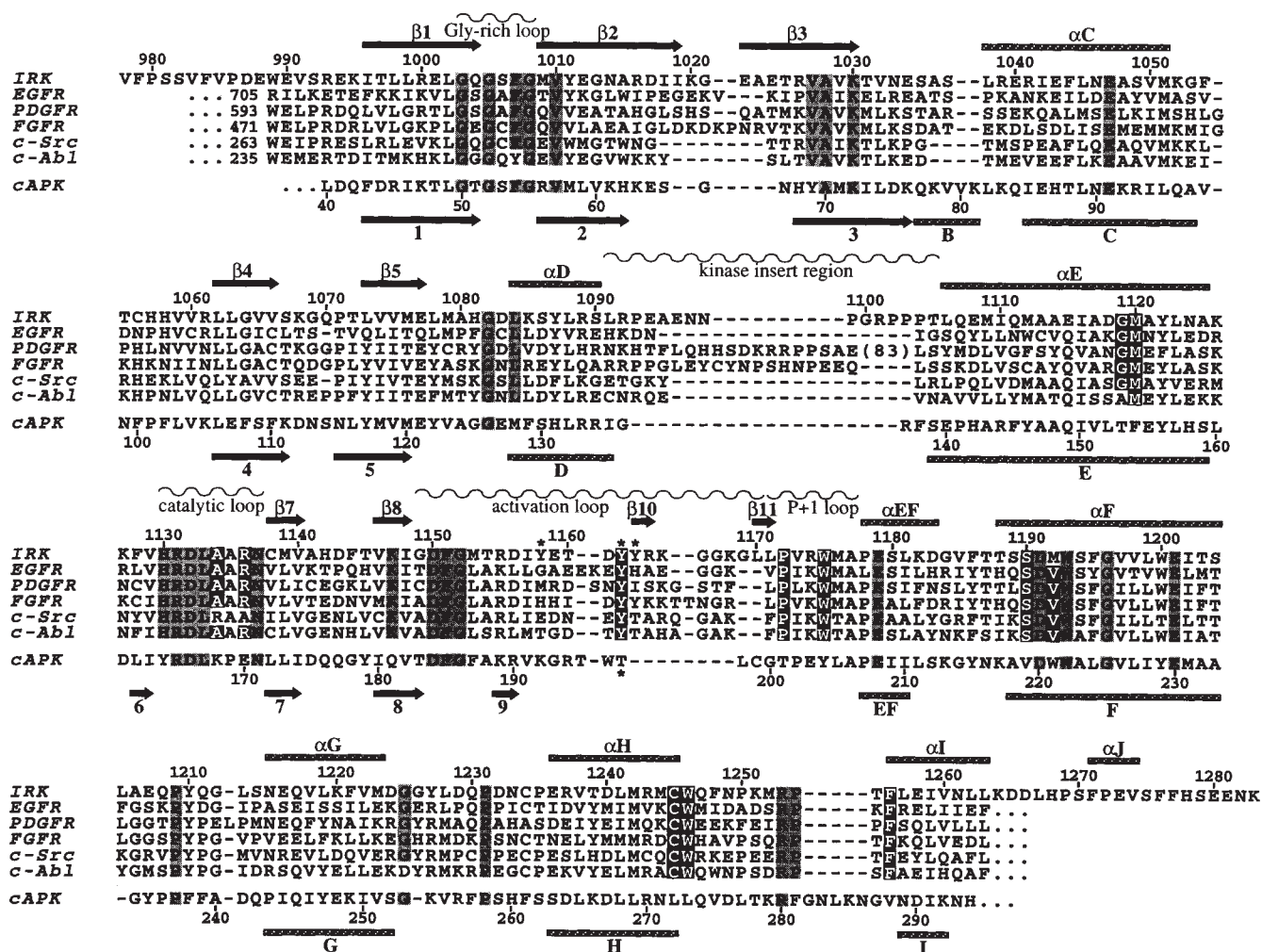


FIG. 3 Structure-based sequence alignment of IRK, CAPK, and the tyrosine kinase domains of the EGF, PDGF (β) and FGF (FLG) receptors and of c-Src and c-Abl. The secondary structure assignments for IRK and CAPK were obtained using the Kabsch and Sander algorithm⁴⁶ as implemented in PROCHECK⁴⁰. A residue is highlighted if found at that position in >90% of the tyrosine kinase sequences listed in Hanks⁴⁷. Of these,

the residues that show conservation in the PSK family as well are shaded, whereas those that are PTK-specific are shown in reverse contrast. The tyrosine autophosphorylation sites in IRK and the essential phosphorylation site in cAPK are marked with an asterisk. No attempt was made to align the PTK sequences between αD and αE and between $\beta 2$ and $\beta 3$.

Phe-Gly accounts for the open conformation of MAPK²¹. The disposition of the lobes in apo IRK places kinase-conserved Lys 1,030, a residue in the N-terminal lobe implicated in ATP binding, a comparatively large distance from the catalytic residues in the C-terminal lobe. In IRK the distance from Lys 1,030 to Asp 1,132, the catalytic base, is 13.7 Å ($N\zeta$ -O $\delta 2$), compared with 12.4 Å in MAPK²¹, 11.2 Å in open-form cAPK, and 7.8 Å in closed-form cAPK.

Active site

The roles of the highly conserved residues in the protein kinase family have been largely elucidated from the crystallographic studies of cAPK complexed with Mg-ATP and PKI^{28,29} (reviewed in refs 30 and 31). The so-called catalytic loop lies between β -strands 6 and 7 in cAPK. In this loop, Asp 166 (Asp 1,132) and Asn 171 (Asn 1,137) are nearly invariant in both the PSK and PTK families, and Lys 168 (Ala 1,134) is highly conserved in the PSK family. Asp 166 is the catalytic base in the phosphotransfer reaction, Lys 168 provides charge neutralization, and Asn 171 is hydrogen-bonded to Asp 166 and is involved in Mg²⁺ coordination. In the PTK family, an arginine is present in the catalytic loop rather than a lysine, either two (Src subfamily) or four residues (all other PTKs; Arg 1,136 in IRK) from the catalytic base (Fig. 3).

One of the most striking features of the apo IRK structure is the presence of Tyr 1,162, a tyrosine that is autophosphorylated upon insulin binding, in the active site of the enzyme, seemingly poised for *cis*-autophosphorylation (Fig. 4a, b). The hydroxyl group of the Tyr 1,162 phenolic ring is hydrogen-bonded to the carboxylate group of the catalytic base, Asp 1,132 (O η -O $\delta 2$: 2.6 Å), and to the guanidinium group of Arg 1,136 (O η -N ϵ : 2.9 Å). The phenolic ring is oriented in part by the pyrrolidine ring of PTK-conserved Pro 1,172. The distance between the centroid of the pyrrolidine ring and the edge of the phenolic ring is 3.6 Å. Arginine 1,136 makes several other contacts: salt bridges to the carboxylate groups of Asp 1,132 and Asp 1,161, and an axial polar interaction³² with the indole ring of Trp 1,175 (N $\eta 1$ -centroid of six-membered ring: 3.1 Å).

The catalytic loop conformations of IRK and cAPK are very similar despite several sequence differences (Fig. 4b). A superposition of the catalytic loops reveals that Arg 1,136N ϵ is the counterpart to Lys 168N ζ in cAPK. The arginine in the catalytic loop of PTKs appears to serve a dual purpose: it provides charge neutralization at the phosphotransfer site (like Lys 168 in cAPK), and makes a hydrogen bond (via N $\eta 2$) with O $\delta 1$ of the catalytic base, which in cAPK is hydrogen-bonded to Thr 201O $\gamma 1$. Either a threonine or serine is found in the PSK

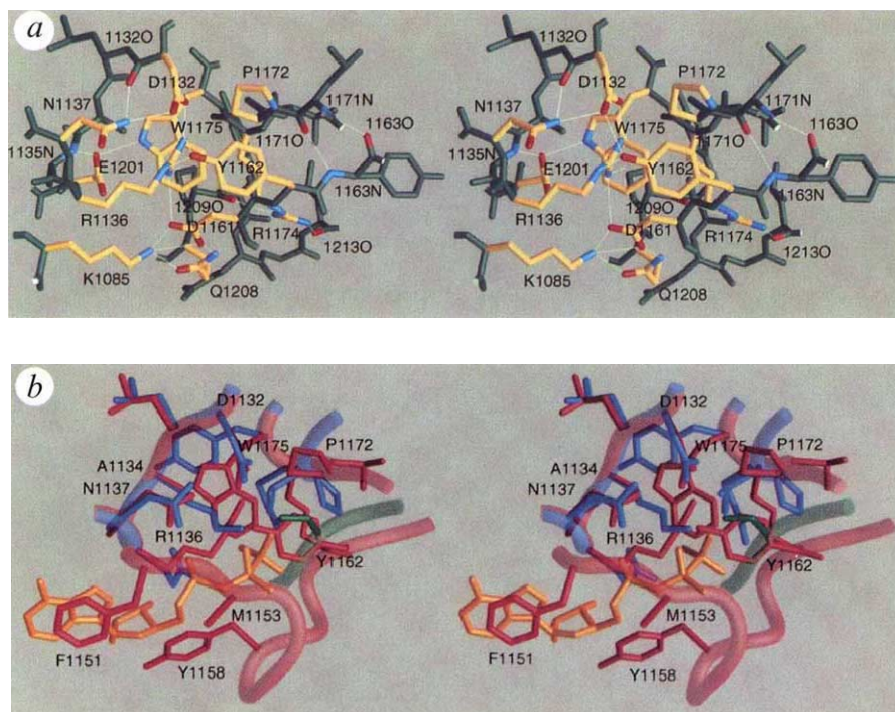


FIG. 4 *a*, Stereo view of the active site of IRK. Highlighted side-chain and main-chain atoms are colour-coded by atom type: carbon, yellow; nitrogen, light blue; oxygen, red. All other atoms are shown in dark green. Hydrogen-bonding interactions are shown by white lines. The view for *a* and *b* is approximately perpendicular to that in Fig. 2*a*, looking along the long axis of the molecule from the N- to the C-terminal lobe. *b*, Stereo view of the active sites of apo IRK and ternary cAPK in which the catalytic loops are superposed. IRK is in red, cAPK in blue, ATP and

PKI from ternary cAPK in orange and green, respectively. Alanine 21 of PKI was changed to serine with the side-chain dihedral angle set to -60° . The labelled IRK residues and the corresponding cAPK residues (in parentheses) are Asp 1,132 (Asp 166), Ala 1,134 (Lys 168), Arg 1,136 (Glu 170), Asn 1,137 (Asn 171), Phe 1,151 (Phe 185), Met 1,153, Tyr 1,158, Tyr 1,162, Pro 1,172 (Thr 201) and Trp 1,175 (Tyr 204). Because of side-chain disorder, only C β of Met 1,153 is shown. Figures drawn with GRASP⁴⁵.

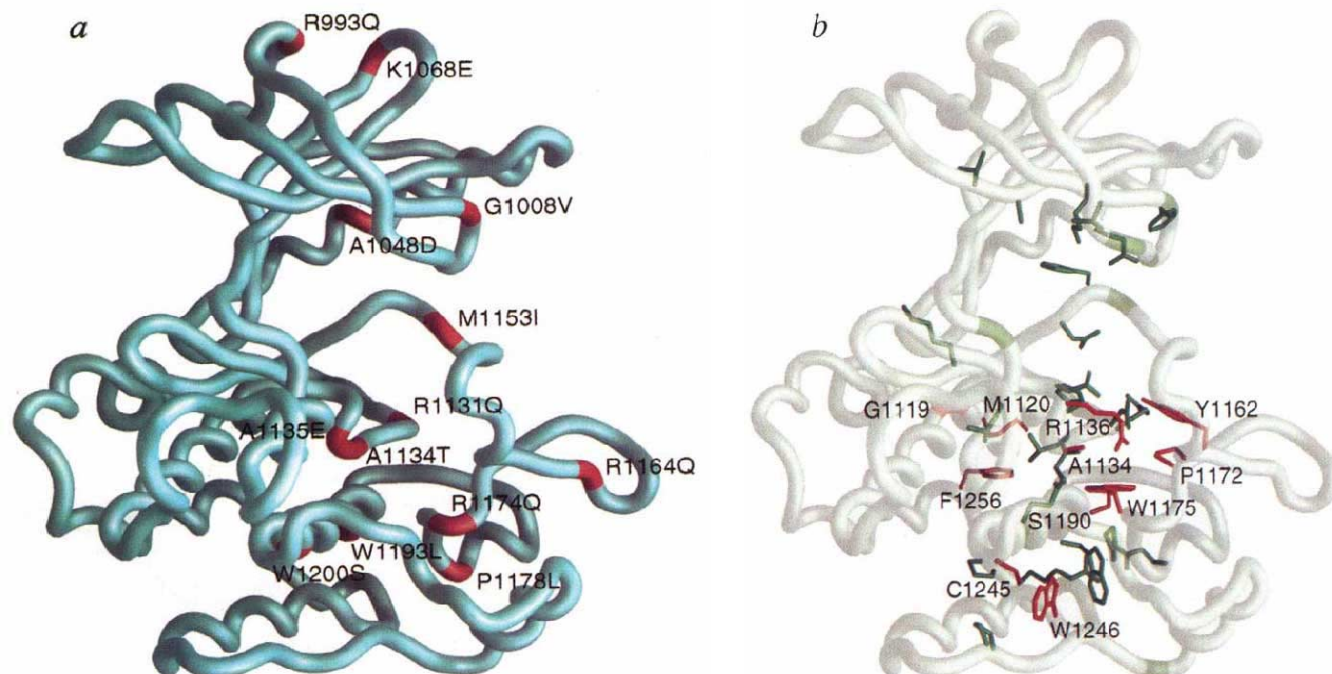


FIG. 5 *a*, Missense mutations in NIDDM patients mapped onto the IRK structure. The mutations, whose positions are shown in red, are Arg 993→Gln, Gly 1,008→Val, Ala 1,048→Asp, Lys 1,068→Glu, Arg 1,131→Gln, Ala 1,134→Thr, Ala 1,135→Glu, Met 1,153→Ile, Arg 1,164→Gln, Arg 1,174→Gln, Pro 1,178→Leu, Trp 1,193→Leu and Trp 1,200→Ser. *b*, Mapping of the highly conserved residues in the PTK

family. In green are residues that show conservation in the PSK family as well, and in red are residues that are PTK-specific (as in Fig. 3). PTK-specific residues are labelled. The positions of conserved glycines are indicated by colouring (green or red) of the backbone representation. Figures drawn with GRASP⁴⁵.

family at cAPK position 201, whereas a proline (Pro 1,172) is found in the PTK family.

Although the hydroxyl group of Tyr 1,162 is in position for phosphotransfer with respect to the catalytic loop residues, the ATP-binding site appears to be inaccessible, precluding *cis*-autophosphorylation of Tyr 1,162. When the catalytic loops of apo IRK and ternary cAPK are superposed, Gly 1,152 and Met 1,153 of the IRK activation loop intersect with (superposed) ATP near the α - and β -phosphates (Fig. 4b). Furthermore, the side chain of Phe 1,151 occupies the hydrophobic pocket in which the adenine ring of ATP sits in ternary cAPK. Val 57 and Leu 173 are situated on either side of the adenine ring in ternary cAPK, and in apo IRK the corresponding residues, Val 1,010 and Met 1,139 (most often a leucine in the PTK family), flank the phenolic ring of Phe 1,151. In both the open and closed forms of cAPK, the phenolic ring of Phe 185 (Phe 1,151) resides in a hydrophobic environment formed by Leu 95 (Met 1,051) and Tyr 164 (His 1,130). The corresponding pocket in the apo IRK structure is largely unoccupied, but is presumably filled by Phe 1,151 in the phosphorylated, activated form of IRK.

Tyrosine substrate selectivity

The polypeptide chain in the near vicinity of Tyr 1,162 appears to mimic the way in which a peptide substrate binds; Tyr 1,162 is an autophosphorylation site (P-site), albeit in *trans* (discussed below), and the interactions of Asp 1,161 (P-1 residue) and Tyr 1,163 (P+1 residue) are consistent with observed substrate specificities for IRK (see below). Therefore, the determinants of tyrosine versus serine/threonine substrate selectivity can be addressed from this apo structure. As seen in the superposition in Fig. 4b, the hydroxyl groups of Tyr 1,162 and Ser 21 of PKI (serine substituted for alanine at the P-site) occupy nearly the same position. Clearly, the hydroxyl group of a serine or threonine side chain at this position in IRK (and in a peptide substrate) would be too short to reach the phosphotransfer site.

The position of the main chain at Tyr 1,162 is determined by the loop that contains residues Leu 1,171 to Ala 1,177, referred to as the P+1 loop (in cAPK, the loop with which the P+1 residue of PKI interacts³¹). Alignments of protein kinase sequences have revealed that the sequence corresponding to Pro 1,172 to Trp 1,175 in IRK is characteristic of the PTK family³³. The two main-chain interactions that govern the distance from the C α atom of Tyr 1,162 to the phosphotransfer site are hydrogen bonds between Tyr 1,163N and Leu 1,171O and between Tyr 1,163O and Leu 1,171N (Fig. 4a). Similar main-chain interactions are present between the P+1 residue of PKI and Gly 200 (Leu 1,171) in ternary cAPK. The active site superposition in Fig. 4b shows that the conformation of the P+1 loop, especially near Pro 1,172 (Thr 201), is an important determinant in substrate selectivity.

The conformation of the IRK P+1 loop is stabilized by a number of interactions. Leu 1,171, Val 1,173 and Met 1,176 form a hydrophobic cluster together with Leu 1,181 and Leu 1,219. Arg 1,174N η 1 and N η 2 are hydrogen-bonded to Pro 1,209O and Leu 1,213O in the connection between α F and α G. An arginine or a lysine is found at position 1,174 in all but the Tyk/Jak subfamily of PTKs. Conserved Trp 1,175 plays a fundamental role in coupling the P+1 loop to the catalytic loop. In addition to the direct, axial polar interaction with Arg 1,136, there is an indirect interaction mediated by Glu 1,201; Glu 1,201O ϵ 2 is hydrogen-bonded to both Trp 1,175N ϵ 1 and Ala 1,135N (Fig. 4a). Glu 1,201 is highly conserved in the PTK family, less so in the PSK family. The primary coupling between the P+1 and catalytic loops of cAPK is the interaction of Thr 201 with Asp 166 and Lys 168, which positions the P+1 loop nearer to the catalytic loop than in IRK, contributing to serine/threonine versus tyrosine substrate selection.

Peptide substrate specificity

The interactions of Asp 1,161 (P-1 residue) and Tyr 1,163 (P+1 residue) with other IRK residues correlates well with observed IRK substrate preferences. Asp 1,161 is salt-bridged to Lys 1,085 and Arg 1,136 and hydrogen-bonded to Gln 1,208 (Fig. 4a). Lys 1,085 and two other positively charged residues, Arg 1,089 and Lys 1,092, all lie along the same face of α D. The latter two residues are potential contacts for negatively charged P-2 and P-3 residues of a peptide substrate. The side chain of Tyr 1,163 (also an autophosphorylation site) resides in a hydrophobic environment formed by Val 1,173 and Leu 1,219, and the hydroxyl group is hydrogen-bonded to the carboxylate group of Glu 1,216. IRS-1 is phosphorylated on at least eight tyrosines by the insulin receptor¹⁴. Four of these tyrosines are preceded by an aspartate or glutamate and all eight are followed by a hydrophobic residue. Furthermore, in a study of PTK substrate specificities using an *in vitro* combinatorial approach, the preferred peptide substrate for IRK contained the sequence Glu-Glu-Glu-Tyr-Met-Met-Met (Z. Songyang and L. Cantley, unpublished results).

IRK mutations in NIDDM

The missense mutations in the tyrosine kinase portion of the insulin receptor gene that have been found in patients with NIDDM¹⁷ are mapped onto the IRK structure in Fig. 5a. Many of these mutations involve replacement of hydrophobic residues with others of different size or with charged residues. At the highly conserved Gly 1,008 position, no room for a valine side chain exists owing to the proximity of the main chain of residues 1,031–1,033. In α C, Ala 1,048C β sits in a hydrophobic pocket which could not accommodate the larger aspartate side chain. Similarly, Ala 1,134 $\rightarrow$ Thr and Ala 1,135 $\rightarrow$ Glu in the catalytic loop and Pro 1,178 $\rightarrow$ Leu at the start of α EF would result in steric clashes. The two tryptophan mutations, Trp 1,193 $\rightarrow$ Leu and Trp 1,200 $\rightarrow$ Ser, apparently destabilize the hydrophobic packing in the C-terminal lobe by introducing side chains of smaller size. These two tryptophans, along with PTK-conserved tryptophans 1,175 and 1,246, form a cluster of four tryptophans in this region of the C-terminal lobe.

Four Arg $\rightarrow$ Gln mutations have been identified. Both N η 1 and N η 2 of the Arg 993 guanidinium group are hydrogen-bonded to the carbonyl oxygen of Pro 1,071 in the loop between β 4 and β 5, thereby stabilizing that region of the N-terminal lobe. Pro 1,071 is a *cis* proline, the only one in the structure, and is reasonably well conserved in the PTK family. In the PTK sequences examined, there is a strong correlation between proline at position 1,071 and arginine at position 993 (Fig. 3). The guanidinium group of Arg 1,131 makes no electrostatic contacts in the present structure, yet this residue is invariant in the PTK family. Based on the cAPK structure, it is expected that in the phosphorylated form of IRK, Arg 1,131 will be salt-bridged to one of the phosphotyrosines in the activation loop (see below). As already discussed, the guanidinium group of Arg 1,174 in the P+1 loop is hydrogen-bonded to Pro 1,209O and Leu 1,213O. Loss of the latter interaction upon substitution with a glutamine probably destabilizes the conformation of the P+1 loop, which is important in substrate positioning. The present structure does not provide an explanation for the adverse effects of Arg 1,164 $\rightarrow$ Gln, nor of Met 1,153 $\rightarrow$ Ile and Lys 1,068 $\rightarrow$ Glu. Insights into the two activation loop mutations are expected from a structure of the phosphorylated form of IRK.

Other conserved residues

A number of highly conserved residues in the PTK family (Figs 3, 5b) do not play a direct role in catalysis, but are important instead in structure stabilization. Many of these residues are also conserved in the PSK family. As in cAPK, Glu 1,179 (Glu 208) is salt-bridged to Arg 1,253 (Arg 280), and Asp 1,191 (Asp 220) is hydrogen-bonded to the backbone amide groups of His 1,130 (Tyr 164) and Arg 1,131 (Arg 165) of the catalytic loop. The

Glu 1,047 (Glu 91) side chain is not salt-bridged to Lys 1,030 (Lys 72), but rather is disordered. Glycines 1,082, 1,152 and 1,225 have backbone torsion angles less favourable for $C\beta$ -containing residues. Gly 1,196 is in an α -helical conformation, but a $C\beta$ atom at this position would result in steric clashes with the side chains of Cys 1,245 and Trp 1,246. The imidazole group of His 1,130 is involved in two electrostatic interactions: N δ 1 is hydrogen-bonded to the backbone amide group of Asp 1,132 (Asp 166), and N ϵ 2 is hydrogen-bonded to a well ordered water molecule which in turn is hydrogen-bonded to Asn 1,137O (Asn 171) and Asp 1,150O δ 2 (Asp 184).

Conserved, PTK-specific residues that play a role in structure stabilization (and not mentioned previously) include Gly 1,119, Met 1,120, Ser 1,190, Cys 1,245 and Phe 1,256. A $C\beta$ atom at Gly 1,119 in α E would result in a steric clash with the carbonyl oxygen of His 1,058. Met 1,120 and Phe 1,256 form part of the hydrophobic environment in which Leu 1,133 of the catalytic loop is situated. The hydroxyl group of Ser 1,190 is hydrogen-bonded to both the amide nitrogen and carbonyl oxygen of Thr 1,187, as well as to a water molecule which is also hydrogen-bonded to Asp 1,191O δ 2. The environment of the Cys 1,245 side chain in α H is hydrophobic, yet it is hydrogen-bonded to the carbonyl oxygen of Leu 1,241, also in α H ($S\gamma$ -O: 3.4 Å). The same hydrogen-bonding interaction for a buried cysteine in an α -helix was observed in myohaemerythrin³⁴.

Cis-inhibition and trans-activation

The apo IRK structure reveals a novel mechanism of autoinhibition in which the hydroxyl group of Tyr 1,162 is bound in the active site. Several PSKs, including protein kinase C, calmodulin-dependent protein kinase II and myosin light-chain kinase, contain within the same polypeptide chain a pseudosubstrate sequence that blocks access to the active site³⁵. Pseudosubstrate sequences resemble exogenous substrate sequences, with the P-site serine or threonine most often replaced by alanine. In contrast, IRK possesses not a pseudosubstrate but a *bona fide* substrate known to be autophosphorylated in response to insulin.

Although evidence for *cis*-autophosphorylation has been reported^{9,10}, our autophosphorylation experiments on IRK (L.W., S.R.H., W.A.H. and L.E., manuscript submitted) and those of others on the insulin receptor cytoplasmic domain¹¹ and intact receptor¹² are consistent with a *trans*-autophosphorylation reaction, including the first phosphorylation event. Moreover, studies on the receptors for EGF, PDGF and fibroblast growth factor (FGF) indicate that autophosphorylation occurs via a *trans* mechanism⁴. Based on these results and the observation that the ATP-binding site is blocked in the unphosphorylated IRK structure, we conclude that the binding of ATP and of 'self' Tyr 1,162 in the active site are mutually exclusive, such that *cis*-autophosphorylation of Tyr 1,162 does not occur to any appreciable extent. Further evidence for this binding exclusivity comes from limited tryptic digestion experiments on IRK which demonstrate that the activation loop is more readily proteolysed in the presence of a non-hydrolysable ATP analogue (data not shown).

A model for insulin receptor activation emerges from the apo IRK and ternary cAPK structures and the biochemical data. In solution an equilibrium exists between two activation loop conformations, one in which Tyr 1,162 is engaged in the active site and both substrate and ATP-binding sites are inaccessible, as seen in the present structure, and the other in which Tyr 1,162 is disengaged and both binding sites are accessible. The former conformation is highly favoured in the unphosphorylated state. When Tyr 1,162 is disengaged, Mg-ATP can bind if it is present. The kinase is then transiently active until Tyr 1,162 returns to the active site. In the absence of insulin, the disposition of the receptor's cytoplasmic domains is such that *trans*-autophosphorylation is prevented. When insulin binds to the α -chains, a change within the quaternary structure of the receptor places the phosphorylation sites of one β -chain within reach of the

active site of the other β -chain, the juxtamembrane tether providing the required flexibility. Intramolecular, *trans*-autophosphorylation can then occur when Tyr 1,162 is disengaged and Mg-ATP is bound. If phosphorylation occurs on an activation loop tyrosine, the loop equilibrium is shifted towards a non-inhibiting conformation—Tyr 1,162 disengaged from the active site—which is stabilized by specific electrostatic interactions involving the phosphotyrosine. The result is a significant increase in kinase activity (up to 200-fold for the tri-phosphorylated state *in vitro*; L.W., S.R.H., W.A.H. and L.E., manuscript submitted).

The activation loop of virtually all PTKs contains from one to three tyrosines, one of which can be readily aligned in sequence with Tyr 1,162 (Fig. 3). Also, Arg 1,131 is invariant in the PTK family and the equivalent arginine in cAPK, Arg 165, is salt-bridged to phosphorylated Thr 197 (P-Thr 197) in the cAPK activation loop. We propose that phosphorylation of Tyr 1,162 is the key step in insulin receptor kinase activation, whereby P-Tyr 1,162 will be salt-bridged to Arg 1,131, stabilizing the non-inhibiting conformation of the activation loop. P-Tyr 1,162 may also interact with Arg 1,155, as this residue is either an arginine or lysine in nearly all PTKs and the corresponding lysine in cAPK (Lys 189) interacts with P-Thr 197. The functions of P-Tyr 1,158 and P-Tyr 1,163 in activation are less clear. A crystal structure of the phosphorylated form of IRK should provide insights into the role of phosphorylation in kinase activation.

We believe that the main aspect of this *cis*-inhibition/*trans*-activation mechanism will apply to many PTK family members. In the unphosphorylated state, the activation loop tyrosine corresponding to Tyr 1,162 will be bound in the active site and, concomitantly, ATP binding will be blocked. Autophosphorylation of this tyrosine in *trans* will stabilize the non-inhibiting conformation of the activation loop through electrostatic interactions between the phosphotyrosine and positively charged residues. Activation of the insulin receptor and its subfamily members will differ from that of other receptor PTKs in the mechanism of signal transduction—ligand-induced intramolecular rearrangement rather than ligand-induced oligomerization. □

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LETTERS TO NATURE

Evidence for magnetic-field-induced anisotropy of the interstellar medium

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TURBULENCE in the interstellar medium transfers energy from parsec-sized regions to much smaller scales, and may be responsible for supporting clouds against gravitational collapse¹. Fluctuations in the electron density, which trace turbulence, occur on scales ranging from 10^6 to $>10^{13}$ cm—the largest range of spatial scales seen in natural turbulence. Despite almost thirty years of study, however, the causes and effects of interstellar turbulence are still poorly understood. Here we present observations of OH masers in the Galactic star-forming complex W49N, which we use as point sources to investigate scattering along the line of sight. The masers' images are elliptical, and aligned roughly perpendicular to the Galactic plane. This alignment suggests that the magnetic field of our Galaxy influences interstellar turbulence^{2,3} by mediating the transfer of energy from large to small spatial scales.

We chose to observe the OH masers in W49N because this 11-kpc line of sight samples interstellar turbulence over a long path through the plane of the Galaxy, and so yields its average properties. Moreover, the expected angular broadening due to scattering of ~ 45 milliarcseconds (mas), as extrapolated from measurements of H₂O masers in W49N (ref. 4) and from OH masers on other lines of sight towards other directions^{5,6}, is fairly well matched to the excellent imaging capabilities of the recently completed Very-Long-Baseline Array (VLBA). Because masers are extremely bright, pointlike objects, their scatter-broadened images, known as scattering disks, are unaffected by their intrinsic structure, or sensitivity limitations⁴. Often many masers lie close together in star-forming regions, so that many close but distinct lines of sight can be studied simultaneously.

We observed the emission from the W49N OH masers at 1,667 MHz on 13 March 1993, using very-long-baseline interferometry at eight VLBA antennas and one antenna of the Very Large Array (VLA). We processed the data with the Mark II processor⁷ of the National Radio Astronomy Observatory to obtain cross-power spectra for each antenna pair, with spectral resolution corresponding to 0.156 km s^{-1} in Doppler velocity. We analysed these data with the AIPS software package. We used observations of unresolved continuum sources and bright maser features to remove instrumental and atmospheric gain and phase variations at each antenna. We analysed, and discuss in this Letter, only frequency channels in which confusing emis-

sion from OH masers in the neighbouring source W49S was absent, and in which masers were detected at five or more times the noise level. We fit elliptical models for scattering disks directly to the measured cross-power spectra, and determined the number and location of maser spots empirically.

The scattering disks of the masers are elongated, and are approximately aligned perpendicular to the Galactic plane. Figure 1 shows the observed distribution of maser spots on the sky. The contours for each maser spot at half of the maximum brightness are shown, magnified eight times for clarity. The mean position angle of the minor axes is parallel to the Galactic plane to within 5° . The alignment of the spots is highly significant, with some evidence for systematic variation across the source. Figure 2 shows the fitted major- and minor-axis sizes, with error bars from post-fit residuals. The axial ratios range from 1.5:1

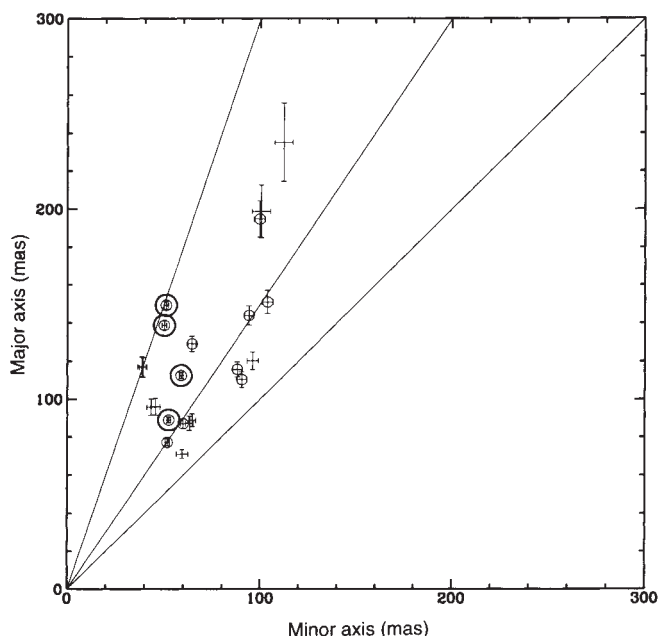


FIG. 1 The observed distribution of maser spots on the sky, showing $\sim 2:1$ elongation, and alignment of minor axes with the Galactic plane, for all maser spots detected at ≥ 5 times the noise level. The average Galactic magnetic field runs along lines of constant Galactic latitude, from lower right to upper left. Contours are 50% of peak brightness (magnified eight times for clarity). We note a systematic variation of the position angles of the major axes with increasing Galactic latitude. The crossed ellipse at the lower right of the figure indicates the (also eight times magnified) effective resolution element, oriented 40° away from the Galactic plane with an axial ratio of 1.4:1, obtained from our many interferometer baselines. (Dec., declination, RA, right ascension; b, Galactic latitude; l, Galactic longitude).

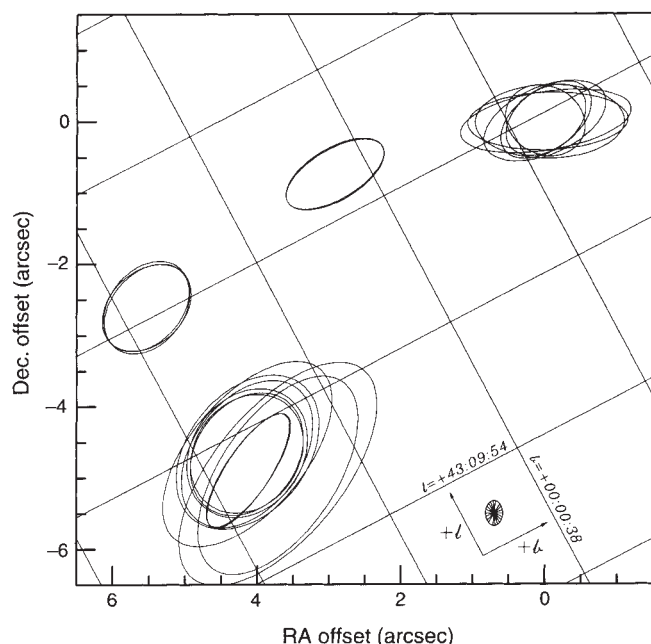


FIG. 2 Scatter plot showing minor and major axes for all maser spots detected at ≥ 5 times the noise level. For each data point, the extent of the cross indicates the formal 1σ error bars of the fitted axis sizes. Maser spots detected at seven or nine times the noise level are indicated by one or two circles around the cross. For reference, lines are drawn for constant axial ratios of 3:1, 2:1 and 1:1.

to 3:1. The distribution of minor axes has a lower limit of 50 mas, about the value expected.

The elongations of the scattering disks and their alignment are due to density fluctuations elongated along a magnetic field. Elongated density fluctuations produce anisotropic scattering by deflecting radiation most strongly perpendicular to their long axes⁸. This anisotropic scattering will be seen only if, along the line of sight, the elongated density fluctuations are aligned. Refraction by large-scale density fluctuations, acting as astigmatic lenses, might elongate individual scattering disks^{9,10}, but are unlikely to align several widely separated scattering disks. In a magnetized plasma, density fluctuations are expected to be elongated parallel to the magnetic field^{2,3} and widely separated scattering disks can be aligned. We thus infer from our observations that the density fluctuations are elongated along an ordered component of the Galactic magnetic field, parallel to the Galactic plane.

We estimated the relative strengths of the magnetic field's random and ordered components using the observed axial ratios of the scattering disks, and models of the Galactic magnetic field and the distribution of scattering material. Our model for the magnetic field consisted of a constant-magnitude component azimuthal in the Galactic plane, B_0 ; and a random component perpendicular to the ordered component, with variance δB . We modelled the scattering material as a uniform distribution of electron-density fluctuations each elongated along the local direction of the model magnetic field by 100:1 or more, as is predicted by theory³. We found that we could reproduce the observed axial ratios with a relative strength of $\delta B/B_0 \approx 0.3$. Other techniques for assessing the relative strengths of these components yield similar values^{11,12}.

Our observations yield the average properties of the turbulence in clumps of strongly scattering material in the interstellar medium¹³⁻¹⁵. The clumps lie near the Galactic plane and are preferentially distributed towards the inner Galaxy. Lines of sight longer than 1 kpc towards the inner Galactic plane invariably intersect these strongly scattering clumps¹⁵, resulting in

apparent sizes up to 100 times larger than that extrapolated for the weakly scattering material smoothly distributed in the interstellar medium¹³⁻¹⁶. Comparisons of scattering strength between sources with small angular separations imply clump sizes of 50–100 pc, and separations of ~ 1 kpc (ref. 17). Apparent sizes of OH maser sources in a variety of directions towards the inner Galactic plane increase linearly with distance, for distances greater than ~ 1 kpc (ref. 5). This implies that our line of sight towards W49N, and other long lines of sight in the Galactic plane, must intersect many strongly scattering clumps, uniformly distributed along the line of sight.

The strongly scattering clumps probably correspond to the extended, low-density envelopes of H II regions observed in low-frequency radio recombination lines^{15,18-20}. Other proposed identifications for the strongly scattering clumps are supernova remnants, their foreshocks, neutral molecular clouds and the hot ionized interstellar medium. Observations of extragalactic sources located near supernova remnants indicate that neither the remnants nor their foreshocks can account for this strongly scattering material^{21,22}. Electron densities in neutral molecular clouds and the hot ionized medium are too low to produce the strong scattering required of the clumps¹⁹. The Galactic distribution of the envelopes of the H II regions, and their estimated sizes of 20–200 pc, are consistent with the postulated distribution and sizes of the strongly scattering clumps. Moreover, plasma parameters of the envelopes, such as electron densities of $1-5 \text{ cm}^{-3}$ and temperatures of $\sim 6,000 \text{ K}$, match those inferred for the clumps^{19,20}. Therefore the strongly scattering clumps seem most likely to correspond to the extended, low-density envelopes of H II regions.

Our observations support recent advances in the theoretical understanding of turbulence in magnetized plasmas. Previous observations have supported the picture of interstellar scattering as arising from turbulence in the interstellar medium¹. Theories have sought to explain the wide range of scales over which this turbulence is observed by analogy to the cascade of turbulent energy from large to small scales seen in neutral, incompressible fluids^{23,24}. Our observations suggest that magnetic fields mediate interactions between spatial scales, promoting a similar cascade, in the interstellar medium. Recent theoretical analyses by Higdon², and Goldreich and Sridhar³ recover the required turbulence, assuming the presence of a strong magnetic field. Those authors also predict that electron density fluctuations will be elongated along the magnetic field lines, resulting in anisotropic scattering, as we observe. We therefore picture the Galactic magnetic field as threading through the extended low-density envelopes of H II regions and elongating the turbulent fluctuations of electron density therein. Those density fluctuations then result in anisotropic scattering disks, aligned over wide angular separations on the sky. □

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Mean age of rifting and volcanism on Venus deduced from impact crater densities

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UNLIKE the extensively cratered highlands of the Moon and Mars, the surface of Venus does not preserve a record of heavy bombardment from the early history of the Solar System^{1–3}. Those craters that are found on Venus appear to be statistically indistinguishable from a random spatial population and rarely show modification by folds, faults and lava flows^{1–3}. Although the volcanic and tectonic history of Venus is still much debated^{2–5}, there is mounting evidence for near-global resurfacing ~300–500 Myr ago^{1,2,6}. Moreover, it has recently been noted that the density of impact craters on large volcanic structures is less than the average crater density of the planet, suggestive of significant activity after the resurfacing event⁷. It is not clear, however, whether these features represent late remnants of the global event or continuing volcanism and tectonism of a still active planet. To address this question, we have used the regional variations in crater density to date volcanoes, rifts and coronae which, based on stratigraphic evidence, clearly post-date the main resurfacing event^{8–11}. The calculated mean ages of 70–125 Myr exclude the possibility that the majority of these features represent the final stages of the global event.

Given that global resurfacing of Venus produced a nearly uniform surface age, the hypothesis of spatial randomness of the craters (Fig. 1a) implies that areas with high or low crater density result solely from the spatially random cratering process. However, several aspects of the crater population are decidedly non-random with respect to geology. Low crater densities occur in regions containing abundant fracture belts and volcanic edifices^{1,3,12}, as well as high proportions of faulted and embayed craters (Fig. 1a)^{1–3}. Crater densities on large volcanoes and coronae are about half that of the global average⁷. These suggestions of nonrandomness can be evaluated quantitatively by comparing a crater database¹³ with high-resolution digital maps of tectonic and volcanic features (Fig. 1b). This approach is appropriate because volcanism and deformation are the only candidate processes for modifying crater density on Venus; erosion is negligible¹⁴. Superimposing crater-density and geological maps (Fig. 1c) reveals that areas of low crater density correlate with concentrations of rifts, coronae and large volcanoes. Furthermore, rifts and coronae have higher proportions of faulted and embayed craters than the plains. These associations suggest that these low-density regions occur, at least partly, as a result of volcanic and tectonic activity, and may possibly be dated using crater density.

Spatial variations in impact crater density are routinely used for dating regions on Mars, Mercury and the Moon, but the method has been considered inappropriate on Venus in light of

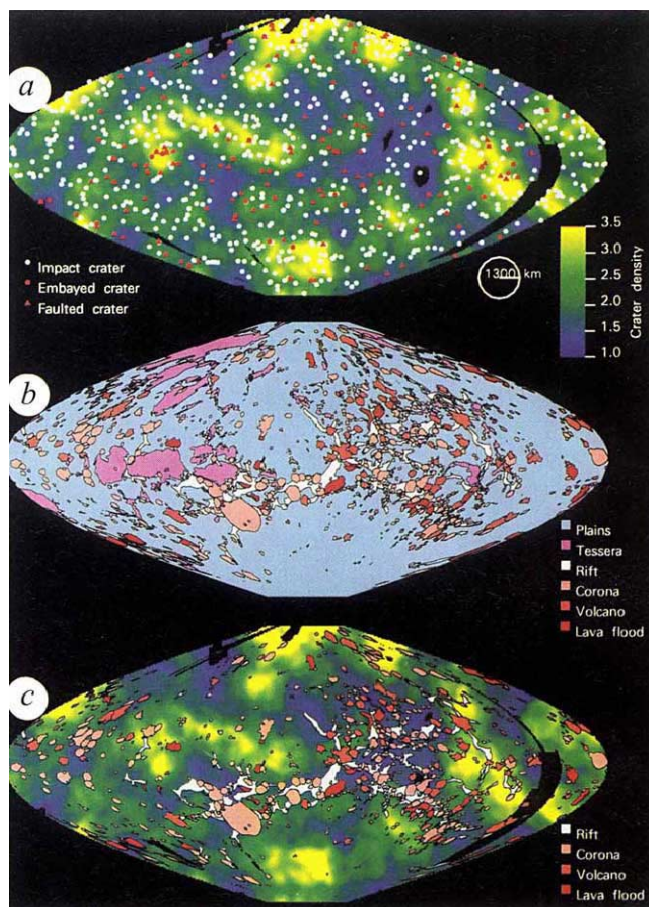


FIG. 1 Maps showing the association of impact-crater density with geological features on Venus. a, Smoothed map of crater density (10^{-6} km^{-2}) and crater locations. The impact craters cannot be distinguished from a spatially random population using the crater data alone, suggesting that a nearly global volcanic resurfacing occurred ~300 Myr ago. b, Tectonic and volcanic features mapped as part of a continuing study of venusian tectonism and volcanism. Mapped units include: $38 \times 10^6 \text{ km}^2$ of tessera (complexly deformed highlands); 150,000 linear km of rifts; coronae and corona-like features (includes 364 coronae, 130 arachnoids, 46 novae and 123 calderas), 128 large volcanoes and 48 flood-type lava flow fields. Many of the corona and volcanic features were first identified by Stofan *et al.*²¹ and Head *et al.*⁹; we have reclassified some features to resolve discrepancies between those databases. About $12 \times 10^6 \text{ km}^2$ of shared area is included in both the rift and corona units. The blue area contains mostly radar-dark plains consisting of overlapping volcanic flows inferred to be products of the global resurfacing^{1,2}. c, Combined map of crater density and geological features. Low-density areas correlate with concentrations of rifts, coronae, volcanoes and lava flow fields, indicating that the craters are not spatially random with respect to geology, and suggesting that volcanism and tectonism since the global resurfacing has reduced the crater density. Maps a–c display the surface of Venus between 82.5° N and 82.5° S latitude, in sinusoidal (equal-area) projection centred at 180° longitude. The density map was generated by counting craters and determining areas within circles of 1,300-km radius. Tectonic and volcanic features were digitally mapped at high resolution using Magellan synthetic aperture radar viewed simultaneously with radar altimetry.

the scarcity and spatial randomness of craters², because low- or high-density areas may occur merely by chance. However, we argue that (1) the lower densities observed in the volcanoes, rifts and coronae result from their youth relative to the plains, and (2) age differences between these features and the plains can be discriminated with reasonable statistical confidence.

First, we test the null hypothesis that the crater density in each terrain equals the global mean density, or $\rho_i = \rho$, implying that their ages equal the global mean age. The terrains are essen-

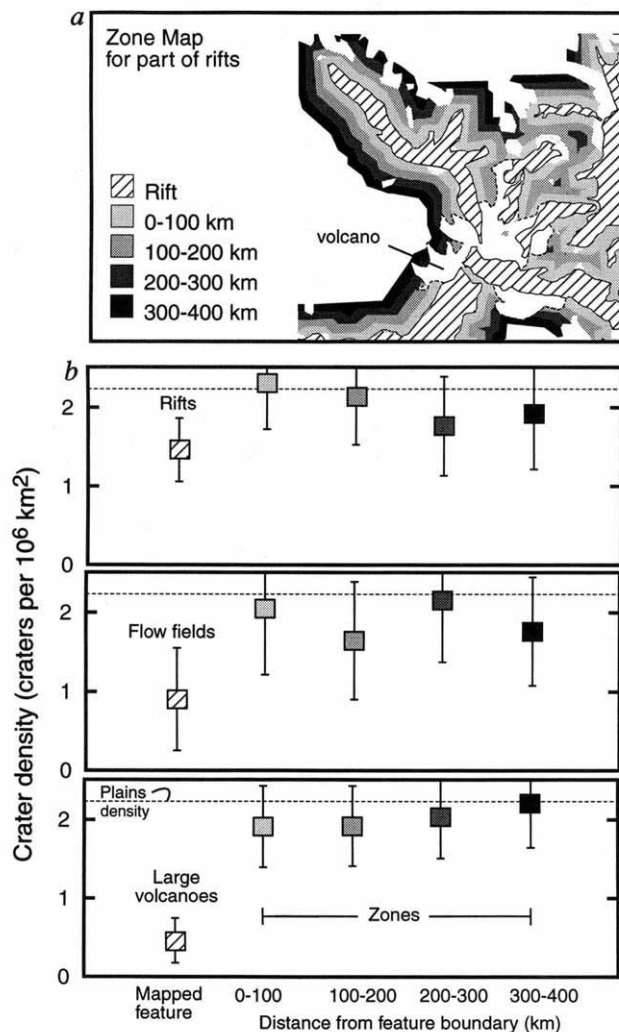


FIG. 2 Crater densities in the plains surrounding rifts, flow fields and large volcanoes. Dating based on crater density alone is difficult because the spatial variation of ages is finer than the resolution defined by a circle of suitable size for reliable crater densities. Using independent geological mapping to define large, genetically related sampling areas results in a much higher dating resolution, as demonstrated by sharp density contrasts between the mapped terrains and the outlying plains. *a*, Partial map of rifts, showing the construction of successive zones in the plains surrounding the rifts. Each zone contains all plains areas lying within a specified range of distance from a rift, and has a minimum area of $15 \times 10^6 \text{ km}^2$. Similar zones are created for the large volcanoes and the lava flood fields to test the resolution of density determinations from geologic sampling. *b*, Crater density changes abruptly between the mapped geological features and the surrounding plains, suggesting that geological mapping of the edges of rifts, flow fields and volcanoes has successfully resolved boundaries of terrains with significantly different ages. Considering that the zones predominantly occur in low-density areas of the crater density map (Fig. 1c), it is even more remarkable that their densities approach the plains average. This observation suggests that the 1,300-km-radius circle used to create the density map is indeed averaging young and old terrains with distinctly different densities. (Error bars, 2σ ; note that the zone width in the flow fields plot is 130 km to maintain sufficient area.)

tially random samples of the global crater population, because they are mapped solely on geological criteria, and the boundaries are independent of crater locations. The crater density, ρ_i , of a mapped unit is the number of craters within it, n_i , divided by its area, A_i . If many random samples of area A_i are taken of N randomly distributed craters, then all n_i will form a binomial distribution which, in theory, may be approximated by a Poisson distribution when A_i is small and N is large¹⁵. We have tested

the density distributions arising from 1,000 randomly generated point sets using $N = 891$ and $A_i = (10-40) \times 10^6 \text{ km}^2$, and find no evidence that this approximation is invalid. In addition, standard deviations of terrain densities determined from 40 Monte Carlo trials, with 891 craters each, fall within $\sim 5\%$ of the predicted Poisson standard deviations $\sigma = (\rho_i/A_i)^{0.5}$. (However, both tests suggest that conclusions regarding flow fields, the geological unit with smallest area, are tentative.) Thus the probability, P , that a terrain density has occurred by chance alone may be estimated using σ , and is less than 0.015 in all cases (Table 1). This result, combined with the spatial association between geology and craters, and the stratigraphic evidence, indicates that difference in age is the most likely reason for the differences in crater density between terrains. We conclude that volcanism and tectonism younger than the plains has subtly modified the crater population so that it is not completely spatially random.

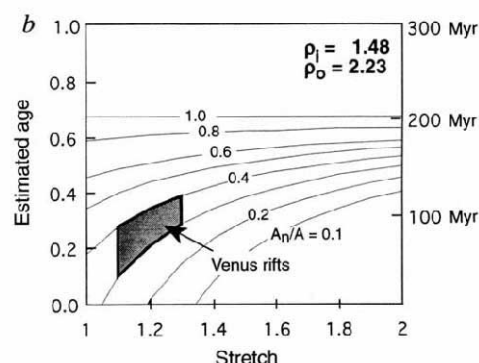
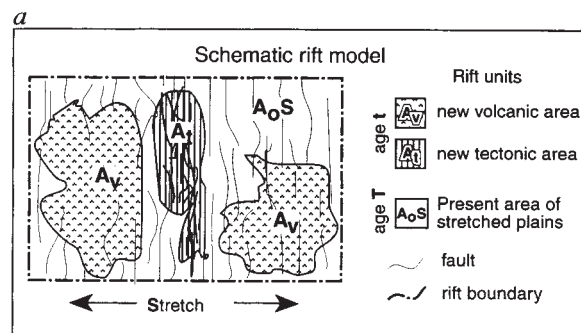
The reliability of the age discrimination between terrains is largely a function of terrain area and the method used to resolve regions of different age. As shown above, the 2σ error of a terrain density may be estimated as $2(\rho_i/A_i)^{0.5}$. If the sample areas A_i are too small, then large errors make the ages meaningless, but large areas are more likely to average densities of young and old surfaces. Thus it is a great advantage to sample areas by geological mapping, using local stratigraphic relationships to map distinct geological features at high resolution. Combining areas of similar features yields a terrain that is sufficiently large for a meaningful age and also contains surfaces of potentially similar age. The abrupt changes in density that occur across our terrain boundaries (Fig. 2) suggest that this sampling method successfully discriminates between terrains of different mean age.

Errors in mapping can strongly affect estimated crater densities, especially for young features adjacent to old plains. For example, if 10% of the volcano map were in fact old plains, then the crater density of the volcanoes could be overestimated by a factor of two. Clearly, the geological criteria used to map features must be carefully defined and applied to obtain meaningful results. Well defined criteria also minimize the potential for map boundaries being inadvertently biased by crater locations during mapping. Bias may also occur when deciding if individual craters should be excluded from the terrain crater count because they predate a structure, based on embayment relationships. Obtaining multiple opinions and assigning half of ambiguous craters to the two classes can reduce such bias.

Having shown that it is reasonable to date large, independently mapped geological terrains, we develop a simple first-order model to estimate the mean age of feature formation in a terrain. Ages are calculated as a fraction of the mean plains age, $T = 300 \text{ Myr}$, which is assumed to reflect the age of global resurfacing (Table 1). The model assumes that cratering is spatially random and rate-constant over the past $T \text{ Myr}$, and that volcanism and tectonism occur simultaneously and instantaneously within a terrain. The second assumption is a substantial idealization, especially as the terrains contain faulted and embayed craters. But most embayed craters occur at the edges of volcanic features, and most faulted craters are found in areas that are not tectonically resurfaced (see below), so the assumption is not unwarranted. The model age is an estimate of the area-weighted mean of the actual mixture of ages in the terrain.

Because volcanic flows can bury craters and create new surface which is exposed to impacts for a fraction of the global resurfacing age, production ages of purely volcanic terrains may be estimated by dividing the observed crater density by the density of the plains (Table 1). But the ages of rifts and coronae cannot be estimated so simply, because these features may contain mildly deformed old plains, as well as young surface created by volcanism or severe tectonism during the formation of the structure (Fig. 3). The density ratio only gives the mean of the crater retention age of the old surface and production age of the young surface. Instead, we develop a simple model that uses estimates of resurfaced area and the tectonic stretch of the old surface to

FIG. 3 Simple dating model and estimated mean rift age. *a*, Model for estimating age of rifts. Such features contain old, moderately deformed plains (A_0S), as well as resurfaced area resulting from severe deformation (A_t) and younger volcanic flows which emanate from the rifts or truncate deformation associated with them (A_v). Tectonism may also change the area of the old surface by extension or shortening, modifying the crater density even if no craters are actually obliterated. The map portrays a schematic rift terrain representing the entire global unit. The rifts form at time t by faulting old plains material and partially resurfacing it; the total new surface area is $A_n = A_v + A_t$. The older surface consists of plains (with age T) that had an original area of A_0 with a crater density ρ_0 , but have been stretched by the amount S (the ratio of the deformed area to the original area). The present total rift area is $A = A_n + A_0S$. We model the present number of craters in the terrain as $n_i = A_0\rho_0 + A_n\rho_0(t/T) + A_0(S-1)\rho_0(t/T)$, in which the first term represents the craters collected over the old plains since time T , the second term includes impacts in the resurfaced area over the shorter time t , and the last term corrects for the stretch. *b*, Estimating age of rifts. Combining equations gives an expression for the estimated age of the rift (as a fraction of the plains age, t/T) in terms of the measurable quantities A , A_n , ρ_t , ρ_0 and S . The graph shows the results of the model for the observed rift (ρ_t) and plains (ρ_0) densities. Ages are given as t/T (left-hand vertical axis) and Myr (right-hand vertical axis). The grey region shows the predicted age of the rifts for our estimated values of new and old surface (A_n/A) and stretch (S). The model was also used to estimate ages of coronae and corona-like features.



find the age of feature formation (Fig. 3). Because the model age is sensitive to these estimates, which are difficult to determine precisely, this model is not ideal for dating tectonic features. It does emphasize, however, the importance of stretch and old surface when interpreting crater densities of tectonic features.

We calculate that large volcanoes, lava flood fields, coronae and rifts have mean ages of 70–125 Myr (Table 1), indicating that a large proportion of the tectonic and volcanic features are substantially younger than the global resurfacing. Such young

deformation and volcanism raises questions in light of proposed models for the tectonic evolution of Venus. Is this activity the result of declining volcanism and tectonism since the end of the global resurfacing^{1,4}. Is it continuing activity of a system that reached a lower equilibrium after the global event? Or is it a resurgence of crustal recycling leading to another global resurfacing¹⁶. Because the ages are similar, and the features tend to be spatially concentrated in areas associated with gravity highs^{9,17,18}, it is possible that these features represent a genetically

TABLE 1 Crater densities and ages of terrains on Venus

Terrain	Area* (10 ⁶ km ²)	Craters	Density (10 ⁻⁶ km ⁻²)	P†	New area A_n/A (%)	Stretch S	Estimated age	
							(t/T)	(Myr)
Global	442.74	891	2.01 ± 0.14					288 (+311, -95) [‡]
Plains	306.15	684	2.23 ± 0.18	0.004				$T = 300$ §
Tesserae	39.54	72	1.82 ± 0.43	0.210			1.01–1.93§	290–570
Volcanoes	19.52	10	0.51 ± 0.32	<10 ⁻⁴			0.23 ± 0.15	69 ± 44
Flows	8.72	8	0.92 ± 0.65	0.011			0.41 ± 0.29	123 ± 87
Rifts	36.45	54	1.48 ± 0.40	0.012	30–40	1.2 ± 0.1	0.27 ± 0.39	81 ± 117
Coronae	38.40	43	1.12 ± 0.34	<10 ⁻⁴	70–100	1.1 ± 0.1	0.28 ± 0.37	83 ± 113
C + CLF	48.54	59	1.13 ± 0.34	<10 ⁻⁴	70–100	1.1 ± 0.1	0.29 ± 0.33	86 ± 100

Ages (t) are given as a fraction of the mean plains age, T , and also in Myr. Ages of volcanoes and flow fields are derived from the ratio of their densities to the plains density. Areas and crater counts were determined using a geographical information system. For rifts and coronae, additional corrections must be made for the proportion of new surface (A_n/A) and the tectonic stretch S of the old surface (see Fig. 3). Age uncertainties reflect the 2σ uncertainty in the density and our estimated uncertainty of A_n/A and S . To determine A_n/A we mapped volcanically and tectonically resurfaced areas in the rifts. We also estimated the amount of resurfaced area in each corona to find the total proportion of new surface: $A_n/A = 70\%$ is our preferred estimate, although it might be as high as 100%. We estimated the stretch based on the mean stretch of 25 deformed craters as determined from crater ellipticity. The most highly deformed craters have stretches near 1.4, so we infer that the mean stretch of the old surface cannot exceed this value, and is probably lower.

* Areas do not sum to global area because of overlaps and gaps in map units.

† Probability of a density less than or equal to the terrain density occurring in a same-area random sample of a spatially random population with the global mean density.

‡ Using cratering rates for craters >35 km in diameter^{2,19}, we calculate the age of our plains unit to be 295 (+311, -95) Myr. But as the uncertainty of the absolute age swamps the difference between the global and plains ages, we simply adopt 300 Myr as the reference age.

§ Ivanov and Basilevsky²⁰ noted a deficit of small craters on tessera, and estimated this crater retention age for tessera, which we include for comparison (having recalculated their age in Myr to reflect $T = 300$ Myr instead of their assumed $T = 500$ Myr). We do not attempt to calculate a tessera age because of the crater deficit and because our model is inappropriate to tessera.

|| Coronae and corona-like features, including coronae, arachnoids, novae and calderas.

linked, recent pulse of activity. Unfortunately, the uncertainties in the ages do not permit a compelling case to be made between continuing, steady activity or resurgent tectonics. Nevertheless, the young mean ages do exclude the possibility that the majority of the features formed during or shortly after the final stages of global resurfacing. Widespread volcanic and tectonic activity has evidently continued on Venus since the end of global resurfacing, and the planet is probably active today. □

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Structure of the 13-K superconductor $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ and the related phase LaNiBN

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THE recent discovery of superconductivity in a number of alloys containing boron and carbon has revived interest in the field of intermetallic superconductors^{1–3}. Although two structural forms have now been identified^{4–6}, most of the new intermetallic superconductors have a crystal structure typified by that of the quaternary alloy $\text{LuNi}_2\text{B}_2\text{C}$, in which layers of rock-salt-structured LuC alternate with nickel boride tetrahedra^{4,5}. The range of superconducting intermetallic compounds was extended recently by the discovery of superconductivity in a system of quaternary boro-nitride alloys, La-Ni-B-N (ref. 7). Here we report the structure of the superconducting phase in this system, $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$, together with that of the related non-superconducting phase, LaNiBN . We find that these two phases are members of a homologous series $(\text{LaN})_n(\text{Ni}_2\text{B}_2)$, with $n=3$ and $n=2$, respectively. Furthermore, our samples contain planar defects that suggest the existence of phases with larger n . Varying the number, n , of LaN layers within this system might thus provide a way to investigate the effect of dimensionality on the superconducting properties of intermetallics.

The specimens were prepared by mixing elemental lanthanum (99.99%), nickel (99.99%) and boron (99.6%) in the appropriate proportions and arc-melting under a nitrogen atmosphere on a water-cooled copper hearth, as described in more detail in ref. 7.

Electron-probe microanalysis (EPMA) was performed on a sample with nominal composition $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_x$, showing a superconducting transition temperature (T_c) of 13.2 K with a high superconducting fraction, using the intensities of the $\text{La } L\alpha$, $\text{Ni } L\alpha$, $\text{B } K\alpha$ and $\text{N } K\alpha$ X-ray emissions in combination with a standard correction for specimen absorption. The specimen contains one major phase, with the approximate composition $\text{La}_{2.8}\text{Ni}_{2.0}\text{B}_{2.2}\text{N}_{3.0}$, suggesting that the phase is $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$.

Electron diffraction and high-resolution electron microscopy (HREM) was performed on one sample with nominal composition $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_x$ (the same as investigated by EPMA) and one with nominal composition $\text{La}_4\text{Ni}_2\text{B}_2\text{N}_x$. Thin specimens for electron microscopy were obtained by crushing in dry iso-propanol (to prevent formation of an amorphous surface layer) and putting a few droplets of this suspension onto a copper grid covered with a carbon-coated holey film. Electron microscopy was performed with a Philips CM30ST-FEG, operating at 300 kV, and equipped with a Link energy-dispersive X-ray (EDX) element analysis system. The electron diffraction patterns were recorded using a Tietz software package and a $1,024 \times 1,024$ pixel Photometrix CCD (charge-coupled device) camera having a dynamic range of 12 bits.

The unit cells and the La/Ni ratios of many crystal fragments were determined by electron diffraction and EDX element analysis. The La/Ni ratios allowed one-to-one correlation with the phases observed by EPMA. $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ was found to have a body-centred tetragonal unit cell with $a=0.373$, $c=2.067$ nm. In the specimen with nominal composition $\text{La}_4\text{Ni}_2\text{B}_2\text{N}_x$, another phase was observed having a La/Ni ratio of 1 and a primitive tetragonal unit cell with $a=0.373$ and $c=0.764$ nm. This La/Ni ratio and the unit cell suggest that this phase is LaNiBN .

Electron diffraction was done on very thin areas (<4 nm thick). This is essential because the interaction of the electrons with the specimen is very strong, resulting in dynamical scattering, which will change the relative intensities of all reflections as a function of the specimen thickness. If such a change has occurred, the intensities of the reflections cannot be used for structure

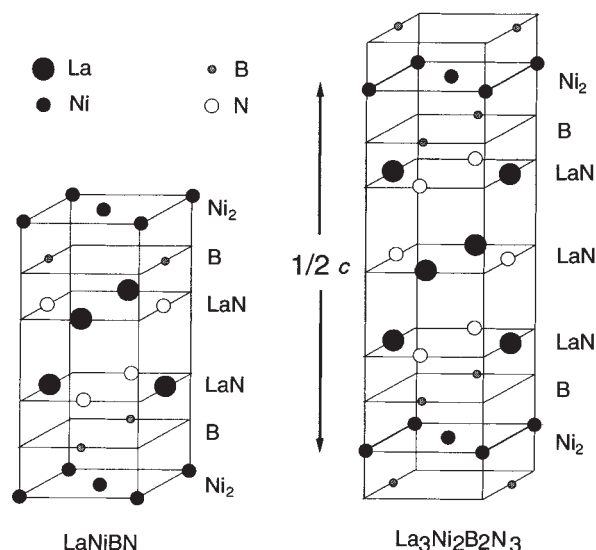


FIG. 1 Schematic representation of the structures of LaNiBN (left) and $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ (right). To enable easy comparison of these two structures and the stacking of the LaN layers, the origins of these two figures do not coincide with the positions of the origins in Table 1.

refinement. To obtain suitable diffraction information, a very thin edge of a crystal was selected using high-resolution mode, after tilting the crystal to give the required orientation. This edge was searched in diffraction mode (with a 10-nm-wide electron beam) for diffraction patterns in which the Friedel pairs (hkl and $\bar{h}\bar{k}\bar{l}$) were almost equal, because quite often the very thin areas are bent from the bulk orientation. Alternatively, the electron beam can be tilted to correct for small misalignment of the diffracting area. To reduce the contamination and amorphization induced by the electron microscope, the specimens were cooled to ~ 100 K. Typical CCD exposure times were 20–100 ms.

A number of diffraction patterns were recorded of $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ in [100] and [110] orientations. The best of these were selected based on the symmetry behaviour of the Friedel pairs and the estimated thickness. The intensities of the reflections were corrected for the misorientation of the crystal fragment and for the curvature of the Ewald sphere. In the latter correction one takes into account the shape of the diffraction spots, which is dependent on the specimen thickness, and the excitation error due to the curvature of the Ewald sphere. Corrected sets for estimated thicknesses of 2.0–4.0 nm (at intervals of 0.5 nm) were made. Refinements using XTAL3.2 (ref. 8) were done with each set of data of the two orientations, each with its own refinable scale factor. Symmetry-related reflections were not averaged, but were used independently. Refinement (63 significant reflections ($>2\sigma$)), started with a model based on the stacking of three LaN layers with a B–Ni₂–B block. The final results are listed in Table 1a. A similar structure determination for a data set of LaNiBN resulted in the parameters given in Table 1b. The resulting structures are shown in Fig. 1.

TABLE 1 X-ray diffraction data from $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ and LaNiBN

(a) $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3^*$				
Isotropic temperature factors	Atomic positional parameters			$U (\text{\AA}^2)$
	x	y	z	
La(1)	0	0	0	0.001 (7)
La(2)	1/2	1/2	0.128 (1)	0.001 (5)
Ni	1/2	0	1/4	0.008 (7)
B	0	0	0.221 (3)	0.23 (11)
N (1)	1/2	1/2	0	0.24 (15)
N(2)	0	0	0.133 (5)	0.12 (6)

(b) LaNiBN [†]				
Isotropic temperature factors	Atomic positional parameters			$U (\text{\AA}^2)$
	x	y	z	
La	1/4	1/4	0.170 (2)	0.003 (4)
Ni	3/4	1/4	1/2	0.07 (1)
B	3/4	3/4	0.44 (2)	0.25 (12)
N	3/4	3/4	0.18 (2)	0.06 (3)

* Space group $I4/mmm$, $a=0.373$, $c=2.067$ nm, 63 significant reflections ($>2\sigma$), $R=11.8\%$, $R_w=8.4\%$ obtained for a Ewald sphere correction for a thickness of 2.0 nm.

† Space group $P4/nmm$, $a=0.373$, $c=0.764$ nm, 69 significant reflections ($>2\sigma$), $R=12.5\%$, $R_w=11.1\%$ obtained for a Ewald sphere correction for a thickness of 3.5 nm.

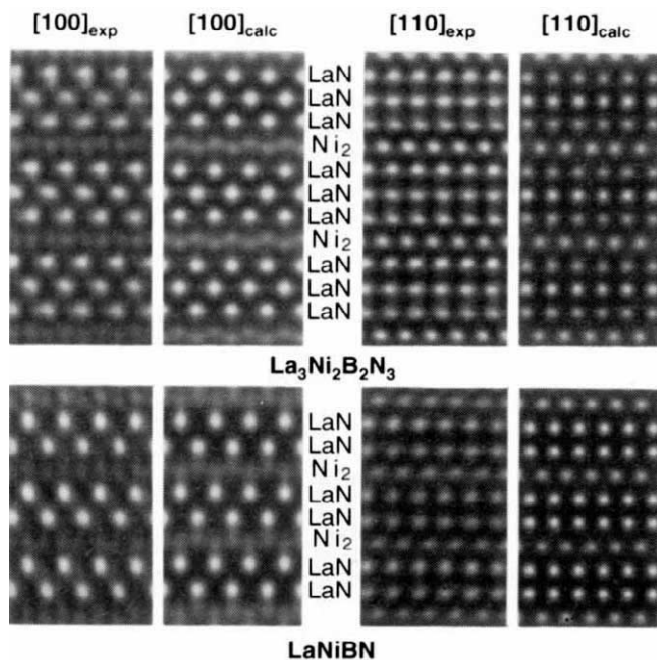


FIG. 2 Experimental and calculated [100] and [110] HREM images of $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ (top row) and LaNiBN (bottom row). The HREM images are averaged over eight unit cells in the horizontal direction. The [100] images show the La and Ni atoms as large and small bright dots respectively. The [110] images show the La and Ni atoms as bright dots of about equal intensity. This equal intensity results from the presence in this viewing direction of twice as many Ni atoms as La atoms. The parameters used in the image calculations are: spherical aberration, 1.3 mm; defocus spread, 5 nm; objective aperture, 10 nm^{-1} ; beam convergence, 0.1 mrad; mechanical vibration, 0.03 nm; specimen thickness, 2 nm; and defocus values ranging from -90 to -100 nm.

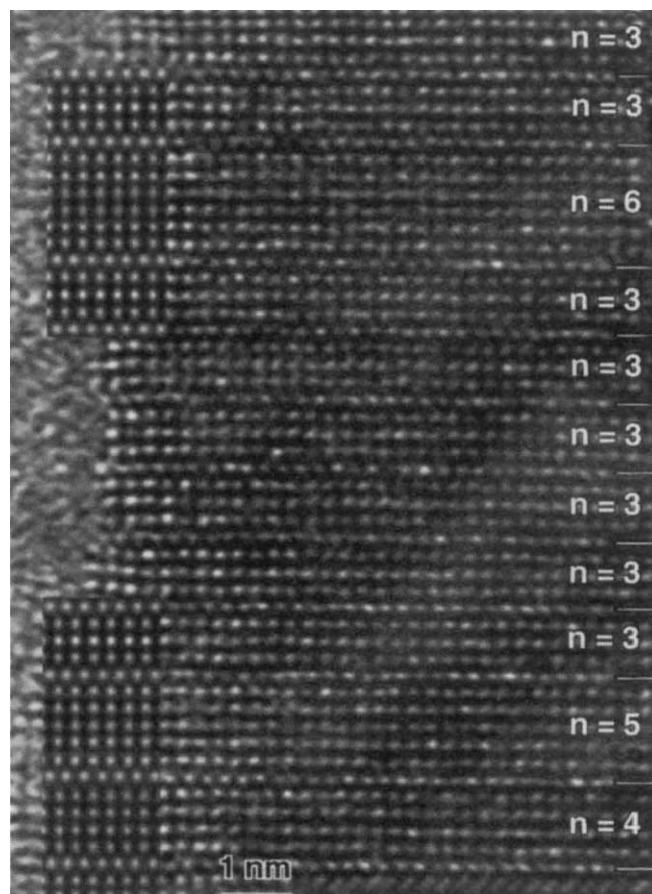


FIG. 3 [110] HREM image of $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ ($n=3$), showing planar defects corresponding to the $n=4$, $n=5$ and $n=6$ members of the series $(\text{LaN})_n(\text{Ni}_2\text{B}_2)$. Calculated images (specimen thickness 2 nm, defocus -90 nm) are shown as insets. Bright dots coincide with La and Ni atoms. Tick marks on the right indicate the Ni planes. Note that a zig-zag stacking of bright dots along the vertical direction occurs only at the Ni planes.

Both refinements result in rather large thermal parameters for N and B. Refinement with diffraction data from slightly thicker areas showed very different thermal parameters and relatively small shifts in the atomic positions. This suggests that these high thermal parameters are mainly due to the dynamical scattering. This agrees with kinematic refinements of diffraction patterns calculated for several thicknesses incorporating dynamical scattering, which show a significant deviation from linear increase of intensities with sample thickness for sample thicknesses >1.5 nm. Alternatively, a surface relaxation could lead to larger thermal parameters.

High-resolution electron micrographs of $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ and LaNiBN in the $[100]$ and $[110]$ orientations are shown in Fig. 2. Image calculations, carried out for the models shown in Fig. 1, give a good agreement with the experimental data.

In many crystals planar defects were observed. An example is shown in Fig. 3. The defects can be related to the presence of extra LaN layers, added to the three LaN layers existing in the $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ structure. In Fig. 3, defects with one, two and three extra LaN layers ($n=4, 5$ and 6 , respectively) can be seen.

The modelling of the structure of the planar defects with more than three LaN can be done based on the evolution of the atomic positions in the series LaNiBN – $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ and the interatomic distances in LaN, which has a rock-salt structure with $a=0.53$ nm. Assuming a perfect rock-salt structure for the LaN layers stacked with a B– Ni_2 –B block as in $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$, a good agreement was obtained for the defect layers, as can be seen from Fig. 3.

The structure of $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ consists of LaN triple layers alternately stacked along the c axis with B– Ni_2 –B blocks. The LaN triple layer forms a rock-salt structure which is also adopted by pure LaN. The La–N distances in $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ are the same as in LaN, as the a axis of $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ is equal to $1/\sqrt{2}$ of the a axis of LaN. The structure of LaNiBN differs from the $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ structure only in the number of LaN layers, being two instead of three. The main interatomic distances in $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ (in nm) are; Ni–B 0.195(5), B–N 0.185(12), La(2)–B 0.328(6) and the La–N distances range from 0.264 to 0.302. For LaNiBN these distances are (in nm); Ni–B 0.193(6), B–N 0.202(18), La(2)–B 0.339(9) and the La–N distances range from 0.264 to 0.279.

The LaN–B– Ni_2 –B–LaN structural unit in $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ and LaNiBN is the same as the LuC –B– Ni_2 –B– LuC unit in $\text{LuNi}_2\text{B}_2\text{C}$ and LuNiBC (ref. 3). This shows that the C atoms can in principle be replaced by N atoms. The structure of LaNiBN is identical to that of LuNiBC (ref. 3). In the system Lu –Ni–B–C another phase occurs, which contains a stacking of four Lu layers: Lu_2NiBC_2 (ref. 4) with a layer sequence (B– Ni_2 –B– LuC – Lu – C_2 – Lu – LuC) $_n$. The structure of Lu_2NiBC_2 differs from that of the $n=4$ member of the homologous series $(\text{LaN})_n(\text{Ni}_2\text{B}_2)$, because it is not a simple stacking of four LuC layers.

Planar defects are regularly observed. They consist of extra LaN layers added to the usual number of LaN layers. The addition of extra LaN layers does not appear to result in strain. This may be because the LaN plane in $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ is exactly the same size as in pure LaN. All phases and planar defects belong to the homologous series $(\text{LaN})_n(\text{Ni}_2\text{B}_2)$. In contrast to the borocarbides of homologous series $(\text{AC})_n(\text{Ni}_2\text{B}_2)$ (where A is a rare-earth element), which form only the $n=1$ member regularly and the $n=2$ member occasionally, the homologous series $(\text{LaN})_n(\text{Ni}_2\text{B}_2)$ is observed as a single crystal for the $n=2$ and $n=3$ members and as planar defects up to the $n=7$ member. Only the $n=1$ member has not been observed, even as a planar defect. This might indicate that this member is not stable. But the absence of any $n=2$ planar defect in a specimen with nominal composition $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_x$ and its abundant presence in a specimen with nominal composition $\text{La}_4\text{Ni}_2\text{B}_2\text{N}_x$, shows that the formation of these phases depends quite strongly on yet unknown synthesis conditions.

The structures reported here suggest that, as with the borocarbides, a whole group of compounds with equivalent structures could be prepared. The rock-salt structure is not only adopted by LaN (unit cell $a=0.53$ nm) but by many other nitrides, for example, SrN ($a=0.54$ nm) and ThN ($a=0.51$ nm). This would allow a variation of the unit-cell dimensions of the basal plane and of the electron count. Furthermore, based on the borocarbides, Ni might possibly be replaced by atoms like Pd and Pt. Finally, the existence of the planar defects corresponding to the $n>3$ members of a homologous series $(\text{LaN})_n(\text{Ni}_2\text{B}_2)$ suggests that other phases might be prepared as single-phase compounds. □

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Relation between metal electronic structure and morphology of metal compounds inside carbon nanotubes

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SEVERAL attempts have been made to fill carbon nanotubes¹ with metals or metallic compounds to obtain nanocomposite materials with potentially interesting properties. Capillary action, predicted² to be a filling mechanism, has been used^{3,4} to encapsulate lead and bismuth in open tubes. Compounds of yttrium⁵, manganese⁶ and gadolinium⁷ have also been encapsulated by formation of the nanotubes in an arc discharge with the metals present *in situ*. Very recently, Tsang *et al.*⁸ showed that oxides of nickel, cobalt, iron and uranium can be encapsulated by opening the tubes and depositing the filling material using wet chemical techniques. Here we report a search for general principles relating to the nature and structure of the filling material, using the arc-discharge method to fill tubes with fifteen metals and/or their compounds: Ti, Cr, Fe, Co, Ni, Cu, Zn, Mo, Pd, Sn, Ta, W, Gd, Dy and Yb. We find that the propensity for forming continuous 'nanowires' throughout the length of the tubes seems to be strongly correlated with the existence of an incomplete electronic shell in the most stable ionic state of the metal. We also find that the interplay between growth of the nanotube and growth of the filling results, in one case, in the formation of an unusual helical filling morphology.

In our arc-discharge synthesis we used as the cathode and anode graphite rods 9 mm in diameter and 42 and 72 mm in

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length respectively. In the anode, a hole 6 mm in diameter was drilled to a depth of 38 mm and filled with a mixture of graphite and metal powders (Table 1). The electric arc and plasma conditions were 100–110 A d.c., 20–30 V, at a helium pressure of 0.6 bar. The distance between electrodes was several millimetres and was manually adjusted to maintain plasma stability; experiment duration depended on plasma stability and was generally 30–60 minutes. The deposit formed on the cathode was ground to a fine powder, ultrasonically dispersed in ethanol and put on a grid for observation using a transmission electron microscope (JEOL 4000 FX at 400 kV).

We chose to investigate 15 elements of various physical and chemical properties to separate the factors having a role in the growth mechanism of nanowires in carbon nanotubes. Table 1 shows the cases in which nanotubes filled with metallic material are abundant relative to hollow nanotubes (Fig. 1e). We can distinguish two kinds of filling. The first type is observed for Cr, Ni, Dy, Yb and Gd. The nanotube is completely filled from its tips (Fig. 1): it has a very regular diameter and the concentric graphite layers are very straight and perfectly aligned along the tube axis as in hollow nanotubes. This morphology resembles that observed for Gd and Y (refs 7 and 9, respectively). These

FIG. 1 Electron micrographs of graphitic nanotubes filled with crystalline metal carbides; Cr (a, e), Ni (b), Dy (c) and Yb (d). Only one part of the filament is shown in a–d, and a low-resolution micrograph is shown in e. The nanotubes are imaged by parallel dark lines, which are the graphite basal planes separated by 0.34 nm. In a and d, the crystalline structure of the metallic carbide is imaged by lattice fringes. In both cases, the fringes are in orientation relationship with the graphitic walls and their spacing is inconsistent with the structure of the pure metals. a, The nanowire probably has a polyhedral cross-section with a large flat region in the projection plane. c, The nanowire has an helicoidal morphology (enlarged in Fig. 4). e, The micrograph is a typical view of chromium-carbide-filled nanotubes, which were observed together with various nanoparticles encapsulated in graphitic shells. Empty tubes were very rarely observed; small arrowheads indicate partially empty tubes. The tube indicated by the big arrowhead is more than 3 μm long and is completely filled: variations in contrast of the filling material are due to orientation variations of the tube on the grid.

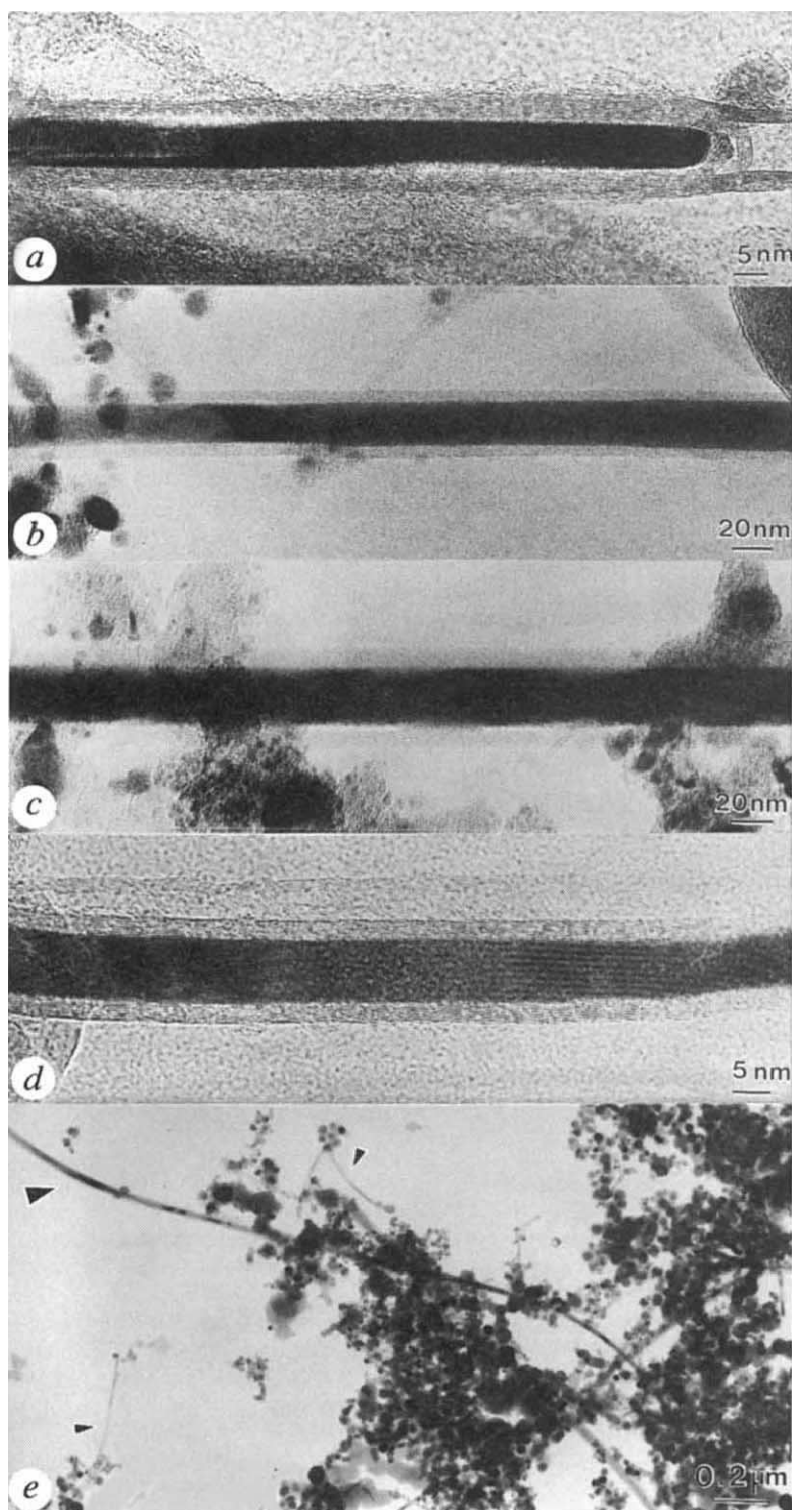


TABLE 1 Main experimental results and electronic configuration of the ions in their most stable state

Elements	Ti	Cr	Fe	Co	Ni	Cu	Zn	Mo	Pd	Sn	Ta	W	Gd	Dy	Yb
Grain size* (μm)	10	5	1	2	10	40	10	10	1	20	40	5	400	400	400
Boiling point (K)	3,562	2,945	3,135	3,201	3,187	2,836	1,180	4,912	3,237	2,876	5,731	5,828	3,539	2,835	1,467
Nanowires†	No	Yes	Yes	Yes	Yes	No	No	No	Yes	No	No	No	Yes	Yes	Yes
Incomplete shell‡	No	Yes	Yes	Yes	Yes	No	No	No	Yes	No	No	No	Yes	Yes	Yes
Length§ (nm)	0	3,000	200	200	1,000	0	0	0	1,000	0	0	0	1,200	600	200
Holes	0	7	5	3	2	0	0	0	2	0	0	0	7	5	1

* Approximate maximum grain size (D_{max}) of the metal powder used. The purity of metals is 99.0–99.95%. The metal powder and the graphite powder ($D_{\text{max}} = 1 \mu\text{m}$, purity 99.4%) were mixed by choosing a graphite/metal volume ratio of roughly the percolation threshold for the metal (70% graphite, 30% metal). After packing the mixture, the volume of the anode hole was filled by approximately 15% metal, 35% graphite and 50% empty space.

† Except for Sn and Mo, nanoparticles encapsulated in polyhedral carbon—similar to those discovered by Ruoff *et al.*¹⁵ (for La) and Murakami *et al.*¹⁶ (for Ta)—were also observed.

‡ Existence of an incomplete electronic shell in the most stable ionic state of the element: Ti^{4+} , Cr^{3+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{1+} , Zn^{2+} , Mo^{6+} , Pd^{2+} , Sn^{4+} , Ta^{5+} , W^{6+} , Gd^{3+} , Dy^{3+} , Yb^{3+} . Note that we do not have any direct information on the ionic state of the metals in the filling compounds.

§ Length of the longest nanowire observed from one or two TEM grids.

|| Number of electronic holes in the incomplete electronic shell for the most stable ionic state.

structures are true metallic nanowires wrapped around by graphite planes extending from 100 nm up to more than 1 μm .

The second kind of filling is observed for Pd, Fe, Co and again for Ni. The fibres resemble those grown by chemical vapour deposition (CVD)^{10,11}. The filling is not complete (Fig. 2): it consists of different encapsulated particles located at the tip (Fig. 2c) and also inside the hollow core at different positions along the nanotube (Fig. 2a, b). Because of the filling discontinuities, the nanotube diameter is not constant along its length and they frequently appear bent, although the graphitic layers remain aligned along the tube axis.

In both types of filling, the particles can extend up to a few hundred nanometres. These filled nanotubes are similar to those obtained for Mn (ref. 6) by the same growth technique. In some cases, the particle at the tip is conical—as in CVD-grown fibres—and the carbon layers are stacked parallel to the side surface of the metallic cone giving rise to conical¹² or bamboo-shaped tubes¹³ (Fig. 2a). We have also observed such conical tubes with a carbide at its tip for W, Ta, Zn and Cu, but no true filled nanotube could be found.

The filling material is a crystalline metallic carbide, except for Co and Cu, which in some cases formed the pure face-centred cubic (f.c.c.) metal. With these two exceptions, electron diffraction data and high-resolution electron micrographs are not consistent with the structure of the pure metals. For instance, for Fe, Ni and Ti we could identify the carbides Fe_3C (cementite), Ni_3C and TiC . Two remarkable situations have been observed for the first kind of filled nanotube (Fig. 3). In Fig. 3a, the Cr-based filling is a single crystal displaying a definite epitaxial relationship with the graphitic layers and with the growth axis (see also Fig. 1d). In Fig. 3b, on the other hand, the filling obtained with Dy is a fine microcrystalline material. A similar microstructure has been found in Y-filled tubes¹⁴.

In the second type of filled nanotube, the filling frequently consists of two or three twinned crystals. In most cases, the tube is cylindrical (Fig. 3a), but some polyhedral wires could be found (Fig. 1a). It is probable that the growth and morphology of the second type of straight and conical filled nanotubes is driven by the metallic particle, which acts as a catalyst, as for CVD fibres^{10,11}. This mechanism seems unlikely, however, for the first

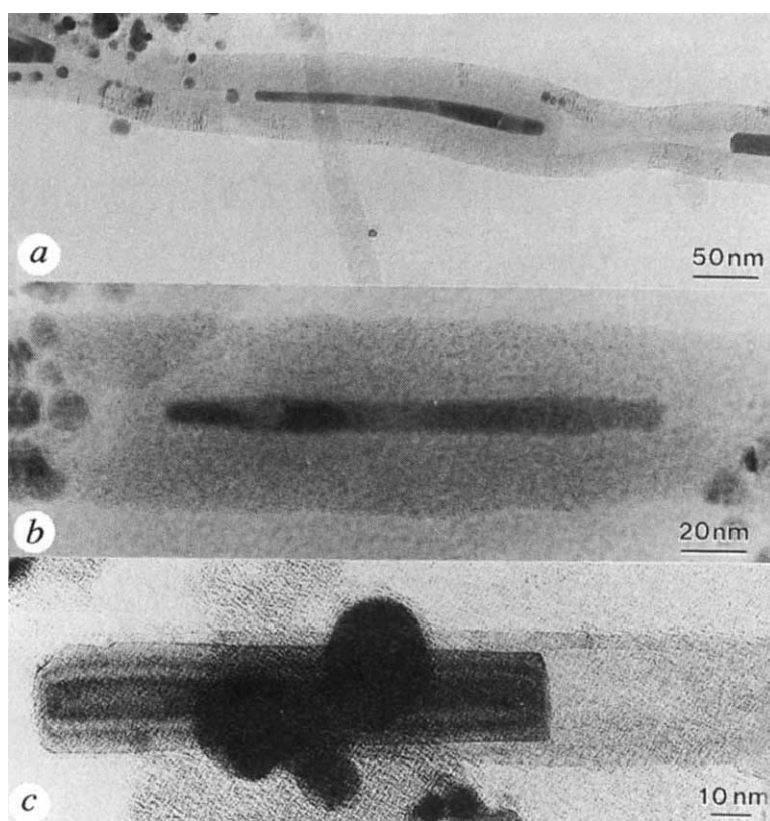


FIG. 2 Electron micrographs of nanotubes partially filled with crystalline metal carbides: Pd (a), Fe (b) and Co (c). In a, the central filament consists of three twinned crystal-lites; only one tip of the filament is capped by graphitic layers, whereas in b the filament is completely encapsulated. In c, the cross-section of the filament is polyhedral. Note the presence of a bamboo-shaped tube in a.

FIG. 3 *a*, Typical high-resolution image of a Cr nanowire. The chromium carbide filament is a single crystal extending over 1 μm , growing epitaxially on the graphitic carbon walls. The cross-section of the filament is probably uniformly cylindrical: graphite fringes progressively diminish from the outside to the inside of the filament. Considering the lattice fringes perpendicular to graphite layers at grazing incidence reveals that they are slightly curved: this indicates the existence of elastic strains at the graphite-carbide interface. *b*, Typical high-resolution image of an Yb nanowire. The ytterbium carbide displays a micro-crystalline micro-structure giving rise, in diffraction, to a characteristic powder pattern. Lattice fringes can be seen for some crystallites.

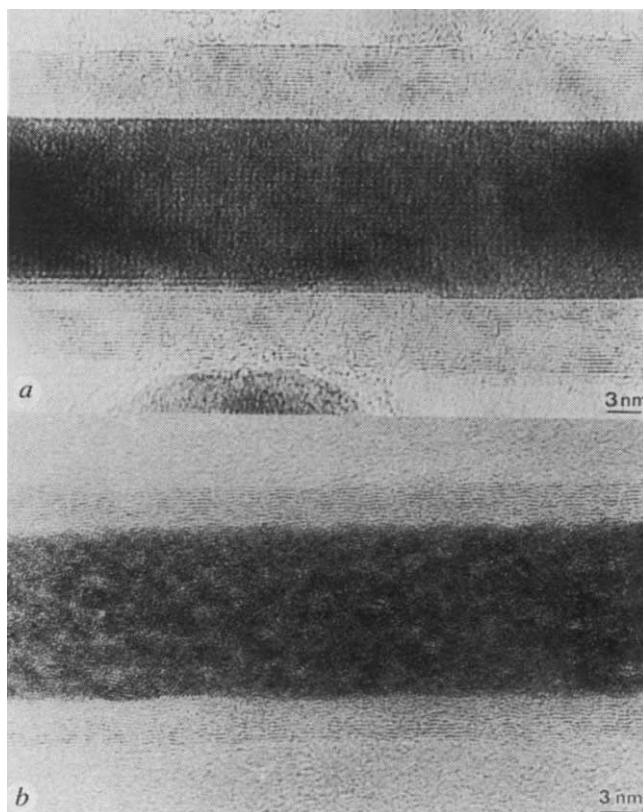
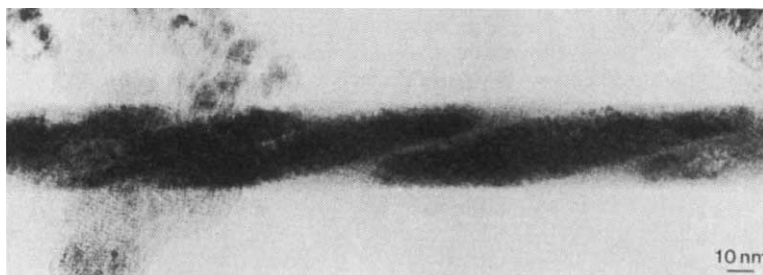


FIG. 4 Bright-field micrograph of a helical Dy nanowire. The image contrast of the carbide strongly suggest that the inner core of the carbon tube filled by the carbide is a helical cavity. This may result from epitaxial growth of the graphite and carbide. The helicity is 10–15°.



type of filled tubes: indeed, some observations indicate that the filamentous morphology of these metallic nanowires is intrinsically determined by the existence of a carbon tube encapsulating it. Figure 3*b* suggests that the carbide is enclosed within the carbon shell while in a liquid state and that it crystallizes in contact with the carbon tube. In Fig. 3*a*, the crystallite is shown growing epitaxially at the graphitic wall. The curvature of the lattice fringes normal to the tube axis indicates the presence of coherency strains at the interface between the carbide and the graphitic layers, adapting the carbide structure to the cylindrical geometry of the tube. These observations suggest that the growths of the nanowire and nanotube are strongly coupled and result from a chemical reaction between the tube and the metallic material. The effect of this coupling is spectacular in the nanowire shown in Fig. 4: the graphitic tube has grown helically and the metallic compound has filled the helical hollow core.

Some clues about the growth mechanism may be derived from the experimental procedure. We can schematically describe the experiment in three steps: first, the boiling of the metal and graphite powder grains on the anode; second, the transfer in the plasma from anode to cathode of metal and carbon ions in an electric and magnetic field; last, the crystalline growth of the nanowires in carbon nanotubes on the cathode.

During the first step, the arc temperature in the vaporization chamber is higher than the boiling temperature of the metals

and graphite: for the 15 elements studied, we find no correlation between the existence of nanowires and the boiling points of the metals (Table 1). For the second step, we can assume that the metal ions are in their most stable oxidation state. If we consider the nature of the electronic shells in that state, we find a striking correlation between the existence of nanowires and the existence of an incomplete electronic shell (Table 1). Furthermore, it seems that the higher the number of electronic holes of the incomplete shell, the greater the length and the quantity of nanowires. In the last step, two properties seem to be important: the abilities of the metals to act as catalysts and to form carbides. It is well-known that the number of electronic holes of the incomplete electronic shell is a fundamental control on these two properties.

We conclude that the existence of an incomplete electronic shell seems to be the crucial factor in the growth mechanism of nanowires in carbon nanotubes. It is striking that, among the 15 investigated metals, Cr and Gd present the largest number of electronic holes and are the two best elements for making very long and numerous metallic nanowires in carbon nanotubes. □

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Effects of forest decline on uptake and leaching of deposited nitrate determined from ^{15}N and ^{18}O measurements

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ATTEMPTS to understand how atmospheric nitrogen deposition affects forest ecosystems^{1,2} have been hampered by the lack of a direct method to trace the fate of the deposited nitrogen. Nitrate originating in the atmosphere has natural abundances of nitrogen and oxygen isotopes that differ measurably from those of soil nitrate³. Here we present an analysis of the isotope ratios of nitrate in spring waters from eight forested watersheds, ranging from apparently healthy spruce plantations to those in decline owing to acidification. We find that for the healthy, slightly declining and limed sites, only 16–30% of the nitrate in spring water originates directly from the atmosphere without being processed in the soil, whereas for more severely damaged sites almost all of the atmospheric nitrate finds its way directly into the spring water. This suggests that acid-induced forest decline significantly inhibits nitrate consumption by soil microorganisms and trees, and that liming to ameliorate soil acidification restores the consumption of atmospheric nitrate. Nevertheless, in limed ecosystems total nitrate output remains high because of internal nitrate production by the ecosystem.

Nitrate concentrations of forest spring waters in northeast Bavaria (Germany) are correlated with forest decline, liming and net nitrification of forest soils^{4,5}. Although nitrification is generally regarded as a prerequisite for nitrate (NO_3^-) leaching², other studies found that the NO_3^- outputs in spring water seemed to be related to nitrogen deposition from air pollution^{6,7}. However, it has previously not been possible to determine whether nitrate in surface waters originated from nitrification processes or from deposited nitrate that was leached through the soil.

The fate of nitrate deposited from the atmosphere into an ecosystem ($\text{NO}_{3\text{atm}}^-$) is determined in general by the interplay between nitrification, (which dilutes atmospheric nitrate), NO_3^- consumption through plant uptake, immobilization by uptake in microbial biomass, and denitrification. Depending on the degree of dilution by nitrification, NO_3^- consumption will decrease the recovery of $\text{NO}_{3\text{atm}}^-$ in spring water. Low recovery of $\text{NO}_{3\text{atm}}^-$ can be due to a low ratio of NO_3^- input to nitrification

and/or a high ratio of NO_3^- consumption to nitrification. High recovery of $\text{NO}_{3\text{atm}}^-$ is possible only if NO_3^- consumption is low compared to nitrification.

These processes can be investigated by using natural isotope ratios of nitrogen ($\delta^{15}\text{N}_{\text{NO}_3^-}$) and oxygen ($\delta^{18}\text{O}_{\text{NO}_3^-}$) in nitrate as tracers to investigate the flux of nitrate through an ecosystem (Fig. 1). Although the interpretation of the nitrogen isotope ratios is problematical because of their narrow range^{8–10}, the oxygen isotope ratios are more informative owing to the large difference in $\delta^{18}\text{O}$ between atmospheric nitrate ($\text{NO}_{3\text{atm}}^-$) and nitrate from microbial nitrification in the soil ($\text{NO}_{3\text{nit}}^-$)¹¹. The $\delta^{18}\text{O}$ values of atmospheric nitrate cover a range³ of $\delta^{18}\text{O}_{\text{NO}_{3\text{atm}}^-} = 52.5\text{--}60.9\text{‰}$; this is different to the $\delta^{18}\text{O}$ atmospheric sulphate which is closer to atmospheric oxygen¹². In contrast, $\delta^{18}\text{O}_{\text{NO}_{3\text{nit}}^-}$ ranges¹¹ from 0.8 to 5.8‰ because one oxygen atom of microbial nitrate originates from atmospheric O_2 ($\delta^{18}\text{O}_{\text{O}_2} = 23.5\text{‰}$) and two atoms from soil water ($\delta^{18}\text{O}_{\text{H}_2\text{O}} = -10.5$ to -3‰)^{11,13}. Denitrification leads to an increase of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in the remaining NO_3^- at a constant ratio^{3,11,14} of 2:1.

This study quantifies the impact of nitrate deposition on nitrate leaching by measuring natural isotope ratios of NO_3^- oxygen and NO_3^- nitrogen in atmospheric deposition as ecosystem input and spring water as ecosystem output. We interpret

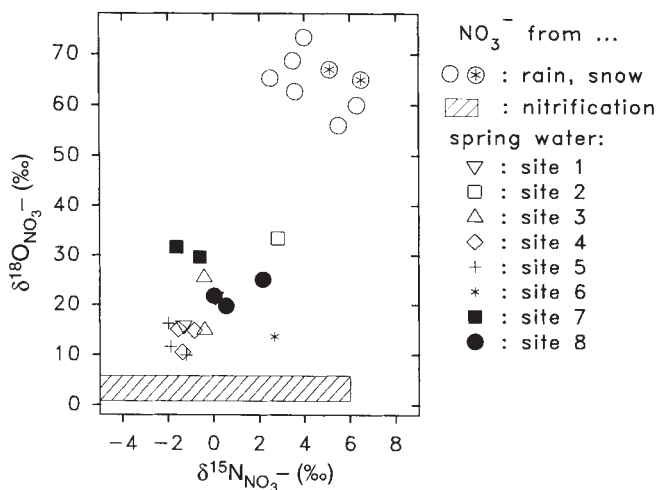


FIG. 1 Natural isotope ratios of nitrate oxygen ($\delta^{18}\text{O}_{\text{NO}_3^-}$) and nitrate nitrogen ($\delta^{15}\text{N}_{\text{NO}_3^-}$) of precipitation nitrate and springwater nitrate from the Fichtelgebirge. Details of sites are given in Table 1. The shaded area represents the range of $\text{NO}_{3\text{nit}}^-$ produced by nitrification of soil nitrogen^{3,11}.

METHODS. Spring water was sampled in November 1991, and March and May 1992. Atmospheric input was collected as rain or snow in autumn and spring below the tree canopy. Natural isotope ratios were analysed following filtration with charcoal, cation exchange (Merck Kationenaustauscher I) and neutralization with KOH. After elimination of sulphate and phosphate by precipitation with BaCl_2 , the water samples were evaporated to dryness. For oxygen isotope analyses aliquots were heated with $\text{Hg}(\text{CN})_2$ in an evacuated sealed tube to 550°C for 6 h. The CO formed was converted to CO_2 by slowly cooling the tube^{3,14}. Carbon dioxide was analysed on a Finnigan MAT delta E gas isotope mass spectrometer. Nitrogen isotope abundances were measured directly from the residue with a system combining an elemental analyser (Heraeus CHN-O Rapid) for Dumas combustion, a Finnigan MAT Trapping Box HT and a Finnigan MAT delta D gas-isotope mass spectrometer⁹. The isotope ratios are reported in delta notation ($\delta^{18}\text{O}$, $\delta^{15}\text{N}$ in ‰) relative to standard mean ocean water or to air nitrogen as the standard⁸. Repeated measurements of standard substances yielded means and standard deviations of $-1.03 \pm 0.12\text{‰}$ for $\delta^{15}\text{N}$ (acetanilide, $n=105$) and $22.44 \pm 0.55\text{‰}$ for $\delta^{18}\text{O}$ (NH_4NO_3 , $n=7$)³.

TABLE 1 Characteristics of sites studied

Site no.	Site condition*	Atmospheric input†		Springwater output	
		NO ₃ ⁻ (mmol m ⁻² yr ⁻¹)	NH ₄ ⁺ (mmol m ⁻² yr ⁻¹)	NO ₃ ⁻ concentration‡ (µmol l ⁻¹)	Total NO ₃ ⁻ output§ (mmol m ⁻² yr ⁻¹)
1	Healthy	46	92	53	29
2	Declining, peaty soil	57	107	43	30
3	Slightly declining	74	108	98	69
4	Healthy	74	81	225	90
5	Slightly declining, limed	45	91	361	90
6	Slightly declining, limed	60	81	191	96
7	Strongly declining	88	96	167	117
8	Strongly declining	36	59	274	137

Site conditions, atmospheric inputs of nitrogen to the watersheds, and NO₃⁻ output characteristics of eight forest springs in the Fichtelgebirge (northeast Bavaria, Germany).

* Definitions: slightly declining, single trees affected by needle yellowing and crown thinning; strongly declining, all trees affected.

† Extrapolated from measurements of throughfall sampled between 15 April and 15 December 1992 with ten funnels per site.

‡ Volume-weighted mean of monthly measurements in 1991 and 1992.

§ Modelled from volume-weighted mean NO₃⁻ concentration and seepage¹⁷.

the observations in relation to soil hydrology and nitrate uptake in these forests.

Eight springs in the Fichtelgebirge (northeast Bavaria; 50° 5' N, 11° 51' E; 700–1,051 m above sea level) were selected from a total of 200 springs under investigation^{4,5}. They represent the observed range of NO₃⁻ concentration in spring water, a wide range of nitrogen inputs and all stages of forest decline (Table 1). All watersheds are dominated by Norway spruce (*Picea abies* [L.] Karst.) forests growing on acid brown earth soils covering granitic and phyllitic bedrock. Locally, hydromorphous peat soils have developed. Springs are mainly fed by interflow with discharge of 0.05–1 l s⁻¹. Nitrogen and sulphur deposition rates greatly exceed critical loads¹⁵. Forest decline due to magnesium deficiency is widespread in the area¹ and liming is widely used for soil amelioration.

We measured $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in atmospheric nitrate. Values of $\delta^{15}\text{N}$ were 2.6–6.3‰ with a mean of 4.3 ± 1.1 ‰; $\delta^{18}\text{O}$ reached values between 60.3 and 73.4‰ with a mean of 64.5 ± 4.8 ‰ (Fig. 1).

Springwater nitrate had $\delta^{15}\text{N}$ values between -2 and +2‰, with $\delta^{18}\text{O}$ between +11 and +33‰ (Fig. 1). Neither nitrification nor denitrification can explain these isotope ratios, as nitrification leads to $\delta^{18}\text{O}_{\text{NO}_3^-}$ values¹¹ between 0.8 and 5.8‰. If the observed isotope ratios were caused by kinetic isotope effects accompanying denitrification of nitrate, a hypothetical NO₃⁻ substrate with $\delta^{15}\text{N}$ between -63 and -19‰ has to be postulated, which is outside the range observed in terrestrial ecosystems¹⁶. However, a small influence of denitrification is possible in samples from sites 2, 6 and 8 (Fig. 1) with $\delta^{15}\text{N}$ of +2‰. These samples have started at $\delta^{15}\text{N}=0$ with an accompanying rise in $\delta^{18}\text{O}$ of +1‰ due to denitrification.

The isotope ratios observed in spring water can only be explained by a contribution of unmodified atmospheric

NO₃⁻ to the NO₃⁻ output. The fraction of atmospheric nitrate in spring water averaged 26%, with 74% originating from nitrification (Table 2). No consistent seasonal trend of the NO₃⁻ fraction was observed. This corresponds to the absence of seasonal trends in NO₃⁻ concentration of these springs.

The $\delta^{18}\text{O}$ values of atmospheric nitrate from summer precipitation has been determined previously³ to be 57.5 ± 2.9 ‰, which is lower than that measured here. Therefore a seasonal variation cannot be excluded, which could affect our data analysis. Nevertheless our calculations are conservative. Assuming atmospheric nitrate to be at $\delta^{18}\text{O}=58$ ‰ instead of 65‰, the calculated fraction of leached atmospheric nitrate would even increase by 2–6%. By using the highest value of atmospheric nitrate (73‰) the fraction of leached atmospheric nitrate would decrease by 1–5%.

Hydrological transport processes in the hilly terrain can influence leaching of NO₃⁻. In the site with peaty waterlogged soils (site 2, Table 1), direct input of NO₃⁻ by surface flow cannot be ruled out as a contributor to the high fraction of NO₃⁻. In all other sites no surface flow occurred, so that NO₃⁻ leaching is regulated only by biological processes in the soil.

The lowest fraction of NO₃⁻ was found in forest stands treated by liming, whereas highest values occurred at sites of forest decline (Table 2). Thus, even in apparently healthy forest stands, nitrate from air pollution may leach through the soil profile without any biological interaction. This implies that adverse effects of high NO₃⁻ outputs from ecosystems, such as cation leaching, soil acidification and eutrophication of surface waters, can be linked directly to NO₃⁻ deposition.

In the healthy, slightly declining or limed sites the amount of NO₃⁻ recovered in spring water is equivalent to 16–30% of the NO₃⁻ input (Table 2, Fig. 2). On these sites NO₃⁻ consumption by plants and soil microflora is still large enough to absorb a high proportion of the deposited nitrate, but not active enough

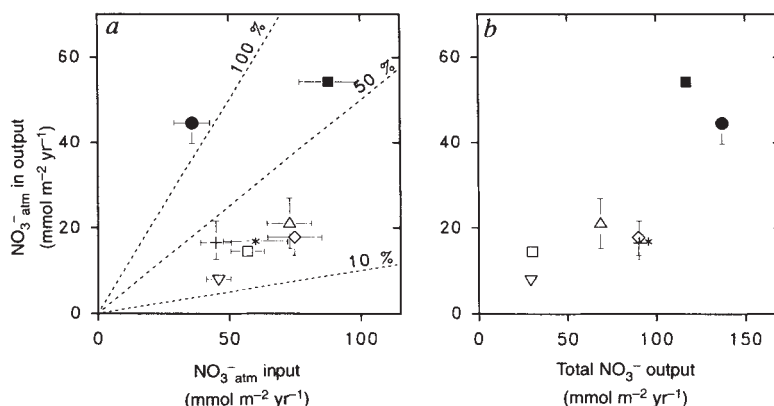


FIG. 2 Absolute amount of NO₃⁻ in springwater output originating from air pollution (NO₃⁻atm in output ± min, max) as a function of atmospheric NO₃⁻ input (± standard error) (a) and total NO₃⁻ output (b). Dashed lines in a indicate percentage recovery of NO₃⁻atm in springwater output (Table 2).

TABLE 2 Nitrate in spring water

Site no.	Output of NO_3^- in spring water		
	Fraction of total NO_3^- output (%)	Absolute flux ($\text{mmol m}^{-2} \text{yr}^{-1}$)	Fraction of NO_3^- input (%)
1	25	8	16
2	46	15	24
3	28	21	26
4	16	18	20
5	15	16	30
6	14	16	23
7	44	54	59
8	30	45	114*

Output of NO_3^- derived from deposition ($\text{NO}_{3\text{atm}}$) in forest spring water in the Fichtelgebirge; means of 1–3 measurements. Because deposited nitrate ($\text{NO}_{3\text{atm}}$) and nitrate originating in the soil ($\text{NO}_{3\text{nit}}$) are the only two sources of nitrate, the fraction of nitrate in spring water originating directly from the atmosphere can be calculated as: $(\delta^{18}\text{O}_{\text{NO}_{3\text{spring}}} - \delta^{18}\text{O}_{\text{NO}_{3\text{nit}}}) / (\delta^{18}\text{O}_{\text{NO}_{3\text{atm}}} - \delta^{18}\text{O}_{\text{NO}_{3\text{nit}}})$ per cent. For $\delta^{18}\text{O}_{\text{NO}_{3\text{atm}}}$, the mean of the measured values (65‰) was used; for $\delta^{18}\text{O}_{\text{NO}_{3\text{nit}}}$, the published value^{3,11} of 3.3‰ was employed in the calculation.

* This value (>100% recovery) could have been caused by errors in the input–output balance, or by temporal $\text{NO}_{3\text{atm}}$ storage in the aquifer.

to metabolize all of it. Generally, one would expect nitrate to be rapidly turned over by microorganisms and vegetation under healthy undisturbed conditions, as well as under disturbed conditions like clear felling or forest decline. But in declining sites with a loss of closed canopy, recovery of $\text{NO}_{3\text{atm}}$ in spring water increases and reaches 59% and 114% of the respective NO_3^- -input (sites 7 and 8, Table 2). An almost complete throughflow of $\text{NO}_{3\text{atm}}$ seems to take place due to a significant reduction of NO_3^- consumption by soil microorganisms and trees.

The results from the limed sites can clarify the possible role of soil microorganisms. Liming results in higher soil pH, and stimulates microbial activity and nitrification in these acidic soils. The limed declining sites (sites 5 and 6) showed reduced recovery of $\text{NO}_{3\text{atm}}$ compared to unlimed declining sites (sites 7 and 8). Thus soil internal NO_3^- cycling by microorganisms is the most sensitive process determining the fate of deposited nitrate. Nevertheless, in limed sites total nitrate output remains at a high level because of ecosystem internal nitrate production.

The fraction of $\text{NO}_{3\text{atm}}$ in nitrate output, as calculated from the natural isotope abundances, still underestimates the total effect of nitrogen deposition on groundwater quality. Even if only a part of the atmospheric nitrate percolates through the soil without microbial interaction, more nitrate in spring water will originate from air pollution, because our measurements do not account for nitrification of deposited ammonium. Atmospheric deposition contains even more ammonium than nitrate (Table 1). Ammonium from deposition will be partially assimilated and nitrified by microorganisms and contribute to nitrate in runoff. It is presently not possible to quantify this fraction from isotopic abundances. □

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Mineral chemistry and density of subducted basaltic crust at lower-mantle pressures

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SUBDUCTED slabs are less dense than the surrounding mantle near the base of the transition zone (~660 km depth) because of the survival of garnet in former basaltic crust: by this depth mantle peridotite has transformed to denser perovskite^{1,2}. The buoyancy of the former basaltic crust may contribute to the observed accumulation or horizontal displacement of many slabs at the base of the transition zone³. Here we report experimental confirmation of the widely held belief that the basaltic crust of slabs eventually transforms to a dense perovskitic lithology, stable in the lower mantle. Synthetic mid-ocean-ridge basalt (MORB) glass subjected to pressures of 45, 80 and 100 GPa in a laser-heated diamond anvil cell transforms to an assemblage of aluminous Mg,Fe silicate perovskite, non-quenchable CaSiO_3 perovskite, stishovite and a sodic, aluminous phase with the Ca-ferrite structure (Fig. 1). Perovskitic MORB is about 0.06 g cm^{-3} more dense than a model lower mantle (PREM) derived from seismological data. Thus even thermally equilibrated perovskitic slabs should encounter no significant hindrance to subduction and convection in the lower mantle.

At depths below 660 km and to at least 800 km, buoyant former MORB is known to comprise neither magnesian silicate perovskite nor magnesio-wüstite, but instead consists of garnet rich in pyrope and almandine endmembers, stishovite, CaSiO_3 perovskite and an aluminous phase with the Ca-ferrite structure². Our preliminary diamond anvil cell experiments on the high-pressure stability limit of garnet in MORB were conducted using a synthetic glass equivalent in composition to the garnet that Irifune and Ringwood² found still survived in MORB at 27 GPa (~800 km depth). This same garnet could be synthesized only below 30–35 GPa. We find no evidence to substantiate predictions⁴ that garnet might survive to depths of ~1,200 km, and instead propose that transformation of subducted MORB to perovskite should be complete by ~900 km depth.

Past attempts to transform synthetic MORB glass to perovskite have been unsuccessful, with the most recent yielding only glass plus metallic iron⁵. These diamond anvil cell experiments essentially recapitulated earlier attempts by Bell and co-workers, in which the recovered samples showed evidence of extensive melting and also contained metallic iron⁶. We do not agree with the suggestion of Bell *et al.* that metallic iron is stabilized as a consequence of the pressure-induced disproportionation of FeO to metal plus ferric iron at pressures above 10 GPa. No such phenomena have subsequently been observed in multi-anvil experiments^{2,7} on MORB glass at pressures up to 27 GPa, nor have we ourselves induced disproportionation in experiments at pressures as high as 100 GPa. However, intense peak temperatures can be attained during the laser heating of a material with intrinsically strong infrared absorption. Thus our preferred

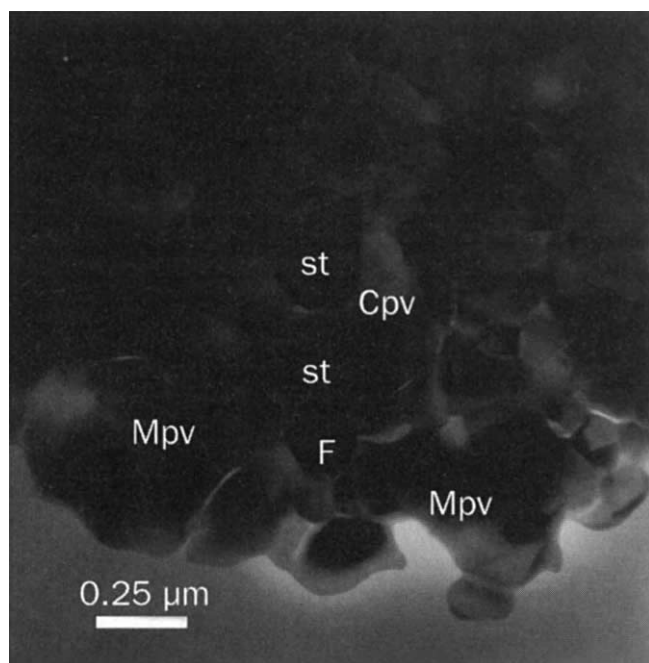


FIG. 1 Transmission electron micrograph of a typical phase assemblage produced from synthetic MORB glass during a diamond anvil cell experiment at 46 GPa. Crystal structures and lattice parameters were obtained by electron diffraction, and phase chemistry obtained by energy dispersive microanalysis of thin foils. Operating conditions of the TEM and analytical details have been described elsewhere²¹. The product assemblage comprises aluminous Mg,Fe-perovskite (Mpv), amorphous calcium silicate perovskite (Cpv), stishovite (st) and a sodic, aluminous phase with the Ca-ferrite structure (F). Neither garnet, magnesio-wüstite nor ' ϵ -MgAl₂O₄' (ref. 25) are found, and there is no sign of melting or reduction. An unstabilized infrared YAG laser attenuated to 10–12 W was used to heat the pressurized sample. Its beam was focused 0.9–1.0 mm beyond the sample through a planoconvex lens of 20 mm focal length. This procedure avoids excessive localized heating of the intrinsically absorbent FeO-rich glass. The energy flux on the sample is about half that necessary to cause melting at 45 GPa, and about one-third that at 100 GPa. If we take Zerr and Boehler's²⁶ solidus for MgSiO₃ perovskite as a rough guide to the MORB solidus, then maximum sample temperatures might lie between 2,000 and 3,000 K. Thus although the laser-heating process gives rise to a temperature profile throughout the sample²⁷, in all probability it encompasses temperatures appropriate for the lower mantle at the pressures of our experiments. Unfortunately, measurement and control of temperature are not technically feasible. Samples (~100 μm in diameter) were contained in steel gaskets and moved in a straight path beneath the laser beam at 5 μm min⁻¹, so as to minimize short-term temperature fluctuations. Pressures measured by the standard ruby fluorescence technique²⁸ are accurate to ± 5 GPa. This same synthetic MORB glass has been used in experiments by other workers^{1,2,5}.

explanation for the stabilization of metallic iron in the aforementioned diamond cell experiments^{5,6} is that temperature-induced reduction of FeO in silicate solid solution has taken place. Having observed that beam energy is absorbed by as little as 8 μm of MORB glass, we modified our laser-heating techniques as described in Fig. 1 legend.

Experiments on synthetic MORB glass at 45, 80 and 100 GPa confirm that transformation to a uniform perovskitic lithology is complete. Table 1 shows that Mg,Fe silicate perovskites possess Mg numbers of ~71, and are thus more FeO-rich than their counterparts, which are stable in the simple system MgO–FeO–SiO₂ (minimum Mg number ~80)^{8,9}. They also contain 16–25 mol% Al₂O₃. Their Mg numbers and aluminous

nature, and the absence of magnesio-wüstite are entirely consistent with our determinations of perovskite phase relations across the composition join pyrope–almandine (Mg_{0.75}Al_{0.5}Si_{0.75}O₃–Fe_{0.75}Al_{0.5}Si_{0.75}O₃). Solid solution of Al₂O₃ greatly expands the compositional stability of Mg,Fe silicate perovskite to at least 75 mol% of the ferrous endmember¹⁰.

Former calcium silicate perovskite in equilibrium with aluminous Mg,Fe silicate perovskite contains only ~2% Al₂O₃, and TiO₂ is strongly partitioned into the latter phase. Stishovite contains a moderate amount (~2.8%) of Al₂O₃ in solid solution but undetectable TiO₂. (The unusually high Al₂O₃ content of stishovite at 80 GPa is tentatively attributed to temperature fluctuations.)

TABLE 1 Compositions and structural formulae of phases synthesized from synthetic MORB glass

	Mg, Al-pv	Ca-pv	st	Ca-ferrite	Mg, Al-pv	Ca-pv	st	Ca-ferrite	Mg, Al-pv*	Ca-pv	st	Ca-ferrite
	45 GPa				80 GPa				100 GPa			
SiO ₂	40.6	56.5	96.5	23.6	43.0	56.7	88.5	26.0	44.7	55.0	96.1	32.4
TiO ₂	2.1	<0.5	<0.5	<0.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Al ₂ O ₃	16.4	2.3	2.8	45.1	20.5	2.1	10.3	45.1	24.0	2.2	2.7	44.1
FeO	17.0	0.8	<0.5	7.8	13.9	2.6	<0.5	9.0	11.7	1.6	<0.5	5.9
MgO	22.6	0.5	<0.5	11.7	20.2	1.4	<0.5	8.9	15.9	0.9	<0.5	7.7
CaO	<0.5	39.9	<0.5	0.9	0.6	36.7	<0.5	0.8	1.4	39.2	<0.5	0.7
Na ₂ O	0.8	<0.5	<0.5	10.7	1.9	<0.5	<0.5	10.3	2.2	1.2	<0.5	9.3
Mg #	70	55		73	72	50		64	71	49		70
Oxygens	3	3	2	4	3	3	2	4	3	3	2	4
Si	0.749	1.05	0.97	0.58	0.78	1.05	0.91	0.64	0.79	1.0	0.97	0.77
Ti	0.03											
Al	0.36	0.05	0.03	1.30	0.44	0.05	0.11	1.30	0.50	0.05	0.03	1.23
Fe	0.26	0.01		0.16	0.21	0.04		0.19	0.17	0.03		0.11
Mg	0.62	0.01		0.43	0.54	0.04		0.33	0.42	0.02		0.27
Ca		0.80		0.02	0.01	0.73		0.02	0.03	0.79		0.02
Na	0.03			0.51	0.07			0.49	0.08	0.04		0.43
Cations	2.05	1.92	1.00	3.02	2.04	1.91	1.02	2.96	1.99	1.96	1.01	2.83

The phases shown here were synthesized in diamond anvil cell experiments at 45, 80 and 100 GPa. Symbols/abbreviations used: pv, perovskite; st, stishovite; Mg # is the Mg number, defined as 100 Mg/(Mg + Fe); n.a., not analysed because most specimens for transmission electron microscopy (TEM) were mounted on Ti metal grids that gave rise to high background signals. Analytical uncertainties are ~1% relative for all elements except Na (1.5%)²¹.

* Minor electron-beam overlap onto neighbouring CaSiO₃ perovskite and Ca-ferrite phase introduces additional uncertainty in this particular analysis.

TABLE 2 Calculated densities of MORB and its constituent phases at 130 GPa

	Al-st	Ca-pv	Mg, Al-pv	Ca-ferrite	MORB
Unit cell volume ($\text{\AA}^3$)	Expanded ~1%				
ρ_0 (g cm^{-3})	4.24	4.25	4.29	4.13	
K_0 (GPa)	314	280	254	225	
K'_0	5	4	4	4	
θ_0	1192	917	957	900	
γ_0	1.7	1.96	1.96	1.3	
Average wt%	11	32	42	15	
ρ 130 GPa, 2,650 K (g cm^{-3})	5.22	5.56	5.73	5.51	5.58

These densities were calculated using the methodology of Ita and Stixrude¹³. Thermoelastic data from the literature^{1,2,13,22-24} provide input parameters. Values for the Ca-ferrite phase are best estimates. Symbols used: ρ_0 , density; K_0 , isothermal bulk modulus; K'_0 , first pressure derivative of K_0 ; θ_0 , Debye temperature (reference value at zero pressure and room temperature); γ_0 , Grüneisen parameter at zero pressure; average wt%, mineralogy derived from mass-balance considerations, using phase chemistry from Table 1.

Most of the Na_2O inventory of MORB is accommodated in the Ca-ferrite phase, with a minor quantity being partitioned into aluminous Mg,Fe silicate perovskite in preference to CaSiO_3 perovskite. Structural formulae considerations indicate that the Ca-ferrite phase comprises significant amounts of the NaAlSiO_4 (ref. 11) and MgAl_2O_4 (ref. 12) endmembers along with partial substitution of 2Al by M + Si (M is Mg, Fe).

Our experiments encompass a pressure range of 55 GPa and temperatures that vary substantially as explained in Fig. 1 legend. In this context, the uniformity of both mineralogy and phase chemistry deserve emphasis. It implies that MORB phase equilibria are relatively insensitive to variations in pressure and temperature, and that the results presented here can be extrapolated throughout the entire lower mantle.

Phase chemistry from Table 1, combined with our best estimates of unit-cell volumes, yields zero-pressure densities for the Ca-ferrite phase, for aluminous stishovite and for aluminous Mg,Fe perovskite (Table 2). The density of former basalt under lower-mantle conditions is calculated from input parameters listed in Table 2 according to procedures described therein. We emphasize that extrapolation of thermoelastic data to pressures corresponding to the base of the lower mantle is an exercise which must necessarily be accompanied by uncertainties of several per cent. It is thus reassuring that the same methodology¹³ applied to a lower mantle of pyrolite/peridotite chemistry and mineralogy yields densities at 130 GPa and 2,650 K that are identical with those of the accepted reference (PREM)¹⁴. The value of 2,650 K reflects current best estimates for temperatures at the base of the lower mantle¹⁵.

New seismic tomographic images¹⁶ reinforce commonsense expectations that thermally retarded slabs should be sufficiently dense to settle through the lower mantle. The relative densities of thermally equilibrated slabs, on the other hand, have been uncertain until now. Table 2 indicates that once MORB transforms to perovskite, it is marginally more dense than a reference PREM mantle by $\sim +0.06 \text{ g cm}^{-3}$. Near the base of the lower mantle (130 GPa, 2,650 K) the density of MORB is 5.58 g cm^{-3} as compared to 5.52 g cm^{-3} for PREM.

We reiterate that the uncertainties in our calculations are of the same magnitude as the density differential between MORB and its surroundings. Nevertheless the unequivocal demonstration that even thermally equilibrated slabs are no longer buoyant below $\sim 900 \text{ km}$ depth has important implications for mantle dynamics. Any slab that succeeds in penetrating $\sim 300 \text{ km}$ below the 660 km discontinuity and transforms to perovskite should encounter no further barrier to entrainment in the convective system of the lower mantle.

Ringwood^{3,17} has proposed that most subducted slabs do not pass unimpeded into the lower mantle, but instead buckle and thicken, forming 'megaliths' at the base of the transition zone. Recent support for his model has come from high-resolution tomographic imaging studies of subduction zones in the western Pacific, which have revealed intriguing structures with appropriate geometries at depths corresponding to the 660 km discontinuity¹⁸. The density relationships reported above mean that the roots of megaliths protruding far enough below the transition zone to transform to perovskite could likewise become entrained in the convective system of the lower mantle.

The dynamic consequences of this intermittent passage of material from the transition zone into the lower mantle may well be profound. Dynamical modelling analyses suggest that cold material from the vicinity of the transition zone at times avalanches downwards into the lower mantle¹⁹. We suggest that former basaltic lithologies in the cool roots of megaliths that protrude well into the lower mantle may survive as metastable garnetite. Their sudden transformation to perovskite with rising temperatures, and consequent loss of buoyancy, may be the very mechanism that initiates these downwelling currents.

Another aspect of Ringwood's model¹⁷ assumes considerable geochemical importance in the present context. He proposed that depleted peridotite on the underside of descending slabs soon reaches thermal equilibrium with its surroundings. These lowermost layers are accordingly eroded and recirculated within the upper mantle. Material reaching the base of the transition zone therefore comprises only the upper parts of the original lithospheric section, namely former basaltic crust plus some underlying harzburgite. The crucial point to recognize is that the proportions of each lithology are such that the bulk composition of the impinging slab should be slightly enriched in basaltic component compared to normal undifferentiated primitive mantle. Thus penetrative slabs and foundering megalith roots, even if thermally equilibrated, could well be significantly more dense than their surroundings in a pyrolite-type lower mantle. Ancient slabs would therefore settle and accumulate at the core-mantle boundary, ultimately to be remobilized as upwelling plumes. Moreover, an overabundance of former basalt in the future source regions of plumes would introduce enriched geochemical and isotopic signatures that would later be inherited by plume-derived basaltic and ultrabasic volcanic suites²⁰. □

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A thalamic nucleus specific for pain and temperature sensation

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THE existence of a posterolateral thalamic relay nucleus for pain and temperature sensation was postulated in 1911, on the basis of the stroke-induced analgesia and thermoaesthesia found paradoxically in patients with thalamic pain syndrome¹. Pain or temperature sensations can be evoked in humans by electrical stimulation in a vaguely defined region of the posterolateral thalamus^{2,3}. Here we use anterograde tracing and single unit recordings to demonstrate that there is a distinct nucleus in the posterior thalamus of the macaque monkey that receives a dense, topographic input from spinothalamic lamina I neurons and in which almost all neurons are nociceptive- or thermoreceptive-specific. Immunohistochemical staining showed that this nucleus is defined by a dense calbindin-positive fibre plexus in the macaque, so we applied the same staining method to sections of human thalamus. We found a nearly identical fibre plexus localized within a distinct nucleus that is cytoarchitectonically homologous to the lamina I relay nucleus in the macaque thalamus. The stereotaxic coordinates of this nucleus and its location relative to the main somatosensory representation fit clinical descriptions of the pain-producing region in humans. We conclude that this is a specific thalamic nucleus for pain and temperature sensation in both monkey and human.

FIG. 1 Colour photomicrographs from the thalamus of the macaque monkey (*Macaca fascicularis*) of: **a**, dense lamina I STT terminations in VMpo labelled with PHA-L (*Phaseolus vulgaris* leucoagglutinin) and Texas red; **b**, topographically distributed trigeminal (orange) and cervical (green) lamina I terminations in VMpo labelled with fluorescent dextrans (only the anterior aspect of the cervical labelling is visible, because it is an anteroposterior topography); **c**, lesions (arrows) delimiting VMpo (the region of darker neurons) between which recordings of nociceptive and thermoreceptive neurons were obtained; and, **d**, PHA-L-labelled lamina I terminations (orange) within the calbindin-positive fibre plexus (green) of VMpo. Scale bars: **a**, **d**, 70 μ m; **b**, 280 μ m; **c**, 590 μ m.

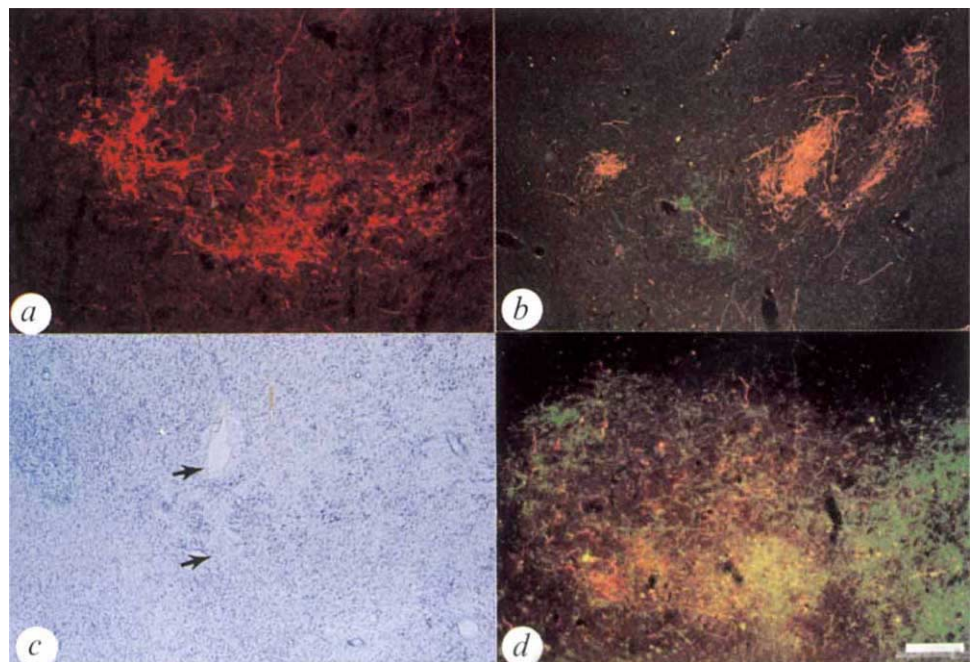
METHODS. Lamina I STT terminations were labelled with PHA-L as described in previous studies of other lamina I projections^{5,18}. In order to demonstrate topography, simultaneous iontophoretic lamina I injections were made at trigeminal and cervical levels, or at cervical and lumbar levels, with FITC- and TRITC-labelled *M*, 10K lysine-fixable dextrans, which can be examined without processing. Single-unit (and cluster) recordings were made under barbiturate anaesthesia as described in previous studies of other nociceptive and thermoreceptive neurons^{7,23,24}. Calbindin

Because the sensations of pain and temperature are unequivocally associated with the spinothalamic tract (STT), understanding their neural mechanisms requires the identification of the thalamic terminations of the nociceptive and thermoreceptive components of the STT. The half of the STT that originates in lamina I of the dorsal horn is of particular interest, because only lamina I receives small-diameter afferent input from all tissues of the body^{4,5}, contains a specific concentration of nociceptive and thermoreceptive STT neurons^{6,7}, and projects ascending STT axons in the middle of the contralateral lateral funiculus (the critical location for pain and temperature sensitivity in humans^{5,8}).

We first examined STT terminations in the thalamus of macaque monkeys in which restricted injections⁷ of high-resolution anterograde tracers had been made into lamina I. We found a dense, compact lamina I STT termination site in the posterior region that is topographically organized anteroposteriorly (Fig. 1a, b). This is a cytoarchitectonically distinct nucleus that we refer to as the posterior part of the ventral medial nucleus (VMpo) because it adjoins anteriorly the basal part of the ventral medial nucleus (VMb) and apparently projects to the middle layers of insular cortex in parallel with VMb (ref. 9, and our unpublished observations). We also observed a sparser topographic projection to the ventral caudal part of the medial dorsal nucleus (MDvc) and weaker input to the ventral posterior group.

We next examined the physiological characteristics of this lamina I STT termination site by recording from single somatosensory neurons located histologically in VMpo (Fig. 1c). We found a compact region containing clusters of nociceptive- or thermoreceptive-specific neurons, posterior to the somatosensory representation of the face. Nearly all (97%) of 87 characterized neurons in VMpo were specifically responsive to noxious or thermal (cold) stimuli (Fig. 2). These VMpo cells had small receptive fields that were topographically organized anteroposteriorly, and their discharge rates graded with stimulus intensity above threshold.

These findings in the macaque monkey indicate that VMpo is a specific thalamic relay nucleus for nociception and thermo-



immunoreactivity was demonstrated with FITC using double-labelling methods²⁵. A monoclonal anti-calbindin (Sigma) was used that produces a slightly different labelling pattern from that reported by others²⁶.

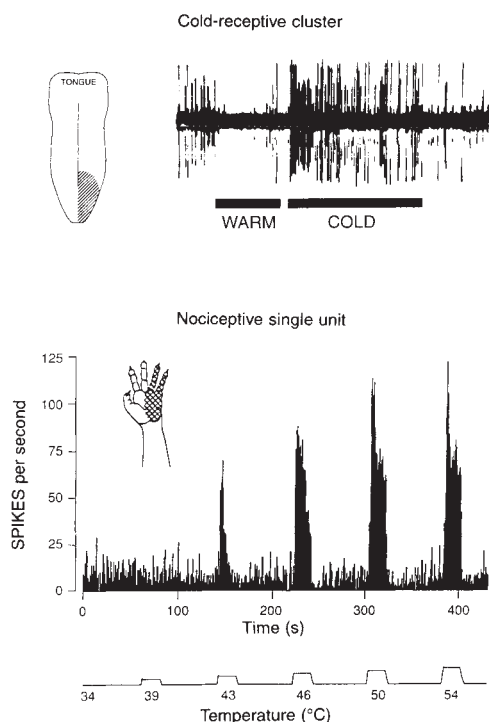


FIG. 2 Examples of the responses of VMpo neurons. The clustered thermoreceptive-specific neurons (top) had ongoing activity that was inhibited by radiant warming, and they were excited by cooling (application of a wet ice cube) and by no other stimuli from a receptive field on the contralateral tongue. The histogram (bottom) shows the graded responses of a single nociceptive-specific neuron to noxious heat pulses applied with a thermoelectric stimulator to the receptive field on the contralateral ulnar hand.

reception. Further, the location of VMpo in monkeys is consistent with the general region in humans in which infarcts can produce analgesia and thermoaesthesia^{1,10}, in which stimulation can evoke painful or thermal sensations^{2,3}, and in which a few nociceptive neurons have been recorded¹¹. As a distinct nucleus has not been identified in this region in the human thalamus, we sought an immunohistochemical marker that could be used to identify VMpo across primates.

Because many lamina I somata are calbindin-immunoreactive¹², we tried an antibody against 28K calbindin and found that VMpo contains a dense calbindin-positive fibre plexus. Anterogradely labelled lamina I STT terminations were coextensive with the calbindin-positive fibre zone in the same or adjacent sections (Fig. 1d), and double-labelled lamina I terminals immunoreactive for calbindin were seen. Although other calbindin-positive regions were observed in the thalamus, these were differentiated from VMpo by intervening unlabelled zones, weaker staining with a different appearance, or the presence of many calbindin-positive cells.

FIG. 3 Photomicrographic pairs from adjacent sections showing the cytoarchitectonic identification of VMpo with the dense calbindin-positive fibre plexus (matching open arrows) in the right posterior thalamus of the macaque monkey (top) and the human (middle) in similar planes of section (monkey, frontal; human, oblique transverse). In the lower panel, VMpo is clearly distinguishable in sagittal sections from the human. Identical vessels are marked by asterisks. The filled arrowheads indicate nearby regions differentiated by calbindin-positive cells. The hole marked with a filled square was made by inserting a pin at the location of the posterior commissure. Scale bars: 1.0 mm, human; 0.55 mm, macaque.

METHODS. The thalamus from normal human autopsy brains was immersion-fixed in 4% paraformaldehyde for 3 weeks and then serial, 50- μ m frozen sections were cut in either the stereotaxic frontal ($n=3$), horizontal ($n=1$) or sagittal ($n=3$) plane, or in an oblique plane midway between frontal and transverse to the brainstem ($n=2$). Standard PAP and ABC/DAB immunohistochemical methods^{25,27} were used to stain 1-in-4 series of sections for thionin, calbindin or other peptides. Endogenous peroxidase activity was suppressed (preincubation in methanol/hydrogen peroxide), and specificity controls (primary antibody omission, antigen preabsorption) were performed. Stereotaxic coordinates were obtained with shrinkage correction based on fresh-brain measurements. Abbreviations²⁸: CM, centré median; MG, medial geniculate n.; Pf, parafascicular n.; Pla, anterior pulvinar n.; VMb, basal part of the ventral medial n.

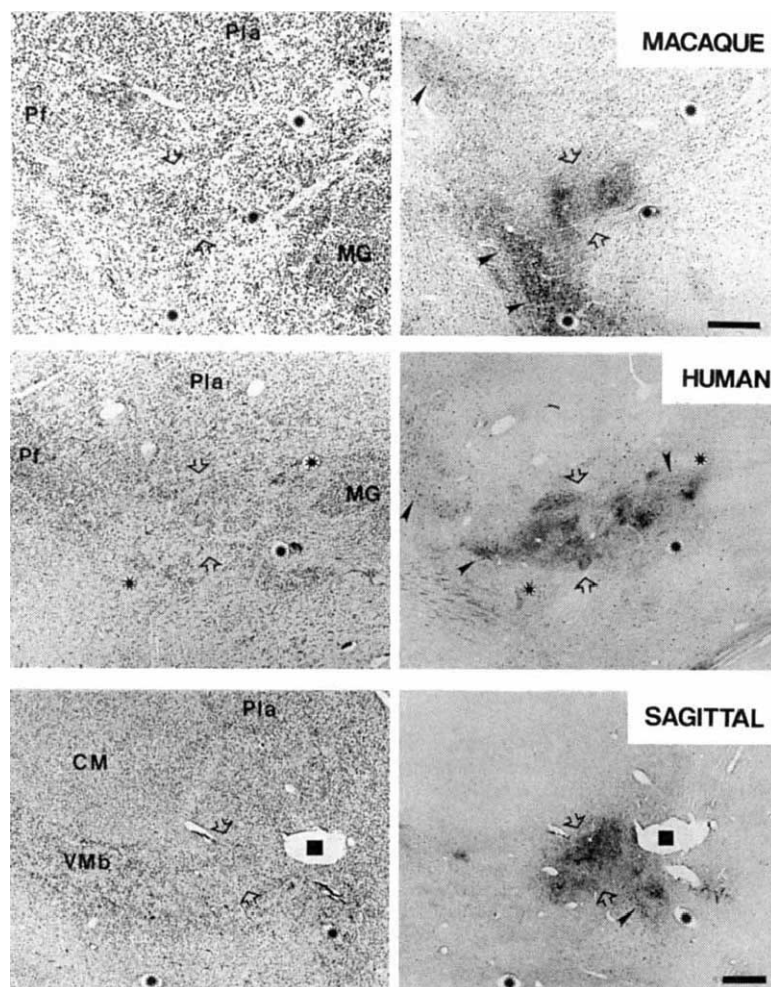




FIG. 4 Drawings of the cytoarchitectonic location of the human VMpo in the 3 standard stereotaxic planes: a, sagittal, L + 14.0 mm; b, frontal, A + 0.5 mm; and c, horizontal, H 0.0. Other abbreviations²⁸: CeM, central medial n.; CL, central lateral n.; H, habenula; L, n. limitans; LD, lateral dorsal n.; LG, lateral geniculate n.; LP, lateral posterior n.; MD, medial dorsal n.; Pc, paracentral n.; Pm, medial pulvinar n.; PO, posterior complex; PV, paraventricular (thalamic) n.; R, reticular n.; SG, supragenulate n.; Sth, subthalamic n.; VA, ventral anterior n.; VLa, anterior part of the ventral lateral n.; VLP, posterior part of the ventral lateral n.; VM, ventral medial n.; VMpo, posterior part of the ventral medial n.; VPI, ventral posterior inferior n.; VPLp, posterior part of the ventral posterior lateral n.; VPM, ventral posterior medial n.

We then stained alternate thalamic sections from nine normal human brains for calbindin or with thionin. We found a dense calbindin-positive fibre plexus in the human posterior thalamus that is unmistakably similar to that found in VMpo in the macaque monkey (Fig. 3), and noted calbindin-positive fibres entering this plexus from the spinal lemniscus. In all planes of section (Fig. 4), this calbindin fibre plexus is localized in a distinct nucleus that is cytoarchitectonically homologous to VMpo in the macaque monkey. It is located dorsomedial to the rostral part of the medial geniculate nucleus, dorsolateral and rostral to the more darkly stained cells of the supragenulate nucleus, and ventral to the anterior pulvinar. Its anterior limit lies at the caudal aspect of the ventral posterior medial nucleus, where medially it adjoins the dorsolateral aspect of the posterior pole of VMB.

Significantly, the average stereotaxic coordinates (extending from about A -0.5 to +2.0, L 12.0 to 16.0 and H -1.0 to +1.0) of the human VMpo and its position just posteroinferior to the main somatosensory thalamus fit the clinical descriptions of the region in which stimulation evokes pain or cold in humans^{2,3}. Nevertheless, this comparison reveals that VMpo extends medially, posteroinferior to the centromedial (CM), beyond where such stimulation attempts have concentrated; more medial exploration should allow observation of the topography of VMpo. The proximity of the human VMpo to CM, in fact, could explain the occasional success obtained years ago with large lesions directed at CM for pain relief¹³. Notably (see below), efficacious CM lesions may have involved both VMpo and the other lamina I pathway through the dorsally adjacent MDvc to the anterior cingulate cortex^{14,16}.

Thus, our observations identify VMpo as a homologous thalamic nucleus in humans and macaque monkeys that relays specific pain and temperature activity. The existence of this nucleus and its apparent cortical projection to the insula provide direct evidence supporting the specific nature of human pain and temperature sensibility and their functional association with the limbic system^{17,18}. These findings demonstrate the structural basis for the strong activation of the posterior thalamus and the insula in positron emission tomography (PET) studies of human pain sensibility^{19,21}. (Additional PET activation in anterior cingulate and SII may result from lamina I input to MDvc and the ventral posterior inferior nucleus, respectively, whereas weaker activation in SI may be due to multireceptive lamina V STT input to the ventral posterior lateral nucleus.) Finally, the identification of VMpo explains the analgesia and thermaesthesia observed in patients with thalamic pain syndrome, which is associated with lesions in this region of thalamus^{1,22}. In this syndrome, a burning dysaesthetic pain is paradoxically referred to the peripheral zone of dense thermaesthesia and analgesia. Our findings are consistent with the proposal^{15,23} that loss of the specific pain and temperature representation in VMpo and the insula results in the disinhibition of the lamina I pathway via MDvc to the anterior cingulate, in remarkable accordance with the essence of Head and Holmes' original proposal¹. □

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Sunburn and p53 in the onset of skin cancer

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SQUAMOUS cell carcinoma of the skin (SCC) can progress by stages: sun-damaged epidermis, with individual disordered keratinocytes; actinic keratosis (AK), spontaneously regressing keratinized patches having aberrant cell differentiation and proliferation; carcinoma *in situ*; SCC and metastasis^{1–3}. To understand how sunlight acts as a carcinogen, we determined the stage at which sunlight mutates the p53 tumour-suppressor gene and identified a function for p53 in skin. The p53 mutations induced by ultraviolet radiation and found in >90% of human SCCs^{4,5} were present in AKs. Inactivating p53 in mouse skin reduced the appearance of sunburn cells⁶, apoptotic keratinocytes generated by overexposure to ultraviolet. Skin thus appears to possess a p53-dependent 'guardian-of-the-tissue' response to DNA damage which aborts precancerous cells. If this response is reduced in a single cell by a prior p53 mutation, sunburn can select for clonal expansion of the p53-mutated cell into the AK. Sunlight can act twice: as tumour initiator and tumour promoter.

To identify the time of sunlight mutagenesis, we first searched for ultraviolet-induced mutations in the p53 gene of AKs. We then looked for evidence indicating whether these mutations had arisen as a prior event in sun-damaged skin or as a later event within the AK. Of 45 actinic keratoses from 24 New England patients, 60% contained a total of 35 p53 mutations (Table 1); eight carried two mutations. Loss of a p53 allele was found in 29% of informative samples. The percentage with mutations may be underestimated as most AKs are not full-thickness lesions. Of the 12 biopsies with the highest proportion of AK to flanking tissue, 75% were mutated.

The base changes found implicated sunlight as the mutagen^{4,5,7}: 89% occurred at adjacent pyrimidines (Table 1) and most were C→T substitutions or CC→TT double-base changes. All coding-region mutations would alter the amino acid. Most patients had multiple AKs in the same region of sun-damaged skin. In five patients, more than one carried a p53 mutation (Table 1). These multiple AKs never shared a common mutation. Each AK's p53-mutant clone thus originated from an independent ultraviolet photon event.

If these p53 mutations arose after the AK, reflecting microscopic SCCs, no AK would contain more than a subclone of p53-mutated cells. But after microdissection, over 80% of the AKs had a mutant sequencing band with 40–100% of the signal, as expected if the mutation was present on one or both alleles in the entire AK sample (Table 1). As the p53 mutations were present throughout most lesions, sunlight evidently acted as a mutagen before or during the clonal expansion of AK, the first clinically apparent lesion in skin cancer development. It is unlikely, but not excluded, that the p53 mutations arose before the AK. A large p53-mutant clone did not precede the multiple AKs, because these AKs had different p53 mutations. If AKs arose within separate small p53-mutant clones, an AK would be surrounded by non-AK cells containing the same p53 mutation. But the frequency of the AK mutation in flanking sun-exposed skin was $\leq 10^{-3}$ in 7 of 8 cases, and never exceeded 10^{-2} (Table 2). It is not excluded that the p53 mutations arise within a clone of cells previously mutated at another gene but not having an AK phenotype.

To investigate the function of p53 in normal skin, wild-type mice were irradiated on the back with varying doses of ultraviolet-B. Twenty-four hours later, sunburn cells⁶ were present in the epidermis (Fig. 1a, b). When stained with haematoxylin-eosin, these had the classic light-microscope appearance of apoptotic cells: pycnotic nuclei and intensely eosinophilic cytoplasm. In p53^{-/-} animals⁸, however, few sunburn cells were induced (Fig. 1c, d). As sunburn cells are often considered 'dyskeratotic', with the eosinophilic cytoplasm presumed to provide evidence of altered keratinization, we confirmed the apoptotic nature of sunburn cells by assaying for DNA strand breaks in keratinocyte nuclei using fluorescent *in situ* end-labelling (FISEL). Figure 1e, f shows that, in wild-type animals, keratinocytes with strand breaks in the nuclear DNA were present 24 h after ultraviolet-B irradiation. In some cells, breaks were localized at the nuclear periphery, resembling the margined chromatin of apoptotic cells seen by electron microscopy. Ultraviolet-B itself did not induce breaks. In wild-type mice, cells with DNA strand breaks appeared with the same time course as apoptotic cells visualized by haematoxylin-eosin (not shown).

The number of sunburn cells in wild-type mice increased with the dose of ultraviolet-B (Fig. 1g). The slope of induction was much less for p53^{-/-} animals. The frequency of apoptosis was intermediate in +/- heterozygous mice (Fig. 1g), as was also observed for γ -irradiated thymocytes^{8,9}. Sunburn cell formation was not just shifted to an earlier time, because at 12 h post-irradiation the frequency in -/- mice was still less than half that in +/- mice (not shown). FISEL-positive cells were also reduced in the p53^{-/-} strain and were intermediate in the heterozygote (not shown); the decrease with decreasing copy number of the p53 gene was statistically significant ($P=0.03$). The doses used in these experiments are ~1 and 2 times the dose required to cause a detectable erythema in mouse or humans¹⁰. For humans, a minimal erythema dose is roughly equivalent to 20 minutes' exposure of untanned skin at noon at middle latitudes. In contrast to the ultraviolet-B response, the apoptotic features of keratinocyte differentiation and the hair cycle appear to be undisturbed in p53^{-/-} mice (K. Stenn, personal communication).

The phenotype of the mutation induced by sunlight should reflect its early occurrence. p53, a transcription factor whose targets include genes involved in cell-cycle regulation¹¹, has been

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proposed to participate in a DNA surveillance pathway that monitors the genome for DNA damage (reviewed in ref. 12). In some cells, this appears to be a 'guardian-of-the-genome' pathway in which DNA damage by ultraviolet light induces p53 protein¹³, leading to transient cell-cycle arrest at a G1 checkpoint^{12,14}. In other cases, however, the endpoint of the DNA surveillance pathway is p53-dependent apoptotic death of the damaged cell^{8,9}. This pathway, which might be termed a 'guardian of the tissue', aborts the aberrant cell rather than restoring its genome. The sunburn cell appears to be the result of this effector step in skin. Whereas the hallmark of sunburn is

erythema, these individual dying cells are microscopic features of sunburned skin⁶ and are distinct from the confluent swollen cells of necrosis. They are seen after irradiation of mammalian skin with ultraviolet-A, -B or -C^{6,10} and can be initiated by DNA damage because their frequency is reduced by reversing cyclobutane pyrimidine dimers in DNA¹⁵. Cells that become sunburn cells seem to be those that were replicating DNA at the time of irradiation⁶. Sunburn cells now appear to be important for preventing skin cancer: they occur in the cell-type of origin for skin tumours, keratinocytes; they result from DNA damage caused by the principal skin carcinogen, ultraviolet radiation;

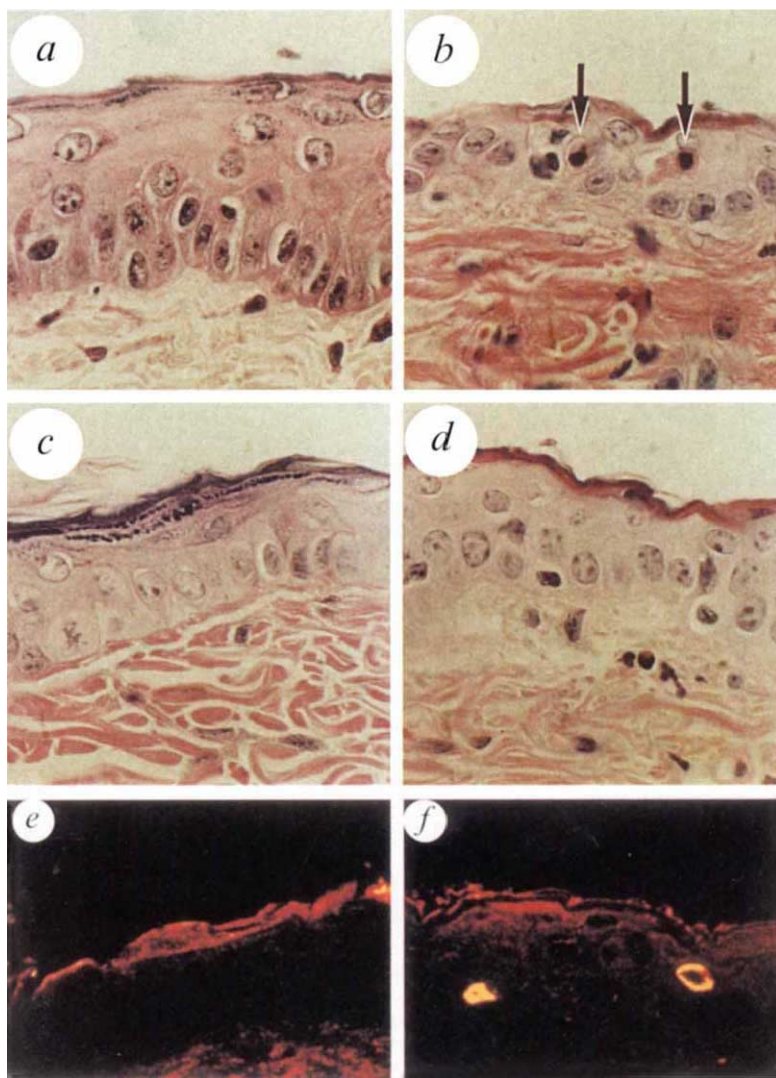
TABLE 1 Sunlight-induced p53 mutations in actinic keratoses

Sample	Site	LOH	Codon	Sequence	Base change	Amino-acid change	Mutation signal intensity (%)
YK 101A	scalp	n.i.	278	tcCt	C → T	Pro → Leu	40
			282	gCcG	C → A	Arg → Leu	30
YK 104A	hand	n.i.	245	gcC*g	C → T	Gly → Ser	35
YK 106A	hand	—	264	atcTa	—T	Leu → ... stop	50
YK 107A	scalp	+	84	gCcc	C → T	Ala → Val	100
YK 108A	scalp	—	205	aTa	T → C	Tyr → Cys	40
YK 109A	shoulder	—	198	tCca	C → A	Glu → stop	50
YK 110A	hand	n.i.	331	cttCa	C → T	Glu → stop	40
YK 112C	neck	—	92	ccCcc	C → T	Pro → Leu	50
			159	gCca	C → T	Ala → Val	50
YK 113A	forehead	—	200 or 201	atTtg	+T	Leu → ... stop	50
			intron 4	aCtg	C → T	effect unknown	30
YK 114A	scalp	n.i.	178	cccCa	C → T	His → Tyr	60
			342	ttcC*g	C → T	Arg → stop	60
YK 115A	hand	+					
YK 115C	forearm	+	238	aCa	C → A	Cys → Phe	40
			241	ttCct	C → T	Ser → Phe	60
YK 116A	arm	n.i.	258	ttCca	C → T	Glu → Lys	50
YK 116B	arm	n.i.	192	ctCa	C → T	Gln → stop	50
YK 116C	arm	n.i.	201–202	tGC*g	GC → TT	Leu-Arg → Phe-Cys	40
			202	gTg	—T	Arg → ... stop	40
			282	acC*g	C → T	Arg → Trp	50
YK 117A	forearm	—	286	tCct	C → A	Glu → stop	60
YK 117B	forearm	—	91	gCca	C → T	Trp → stop	30
			179	acCa	C → T	His → Tyr	50
YK 118C	forearm	n.i.	279	tcCca	C → T	Gly → Glu	30
YK 119B		n.i.	intron 2	tcCa	C → T	effect unknown	40
YK 120A		n.i.	128	ccCc	C → T	Pro → Ser	40
YK 120B		+	247–248	aCC*g	CC → TT	Asn-Arg → Asn-Trp	90
YK 121A		+	248	acC*g	C → T	Arg → Trp	50
YK 121B		—	245	gcC*g	C → T	Gly → Ser	30
YK 122A	cheek	+	135–136	gCCa	CC → AT	Cys-Gln → stop-stop	100
YK 123A	cheek	—	245	gCc*g	C → T	Gly → Asp	40
YK 124A	hand	—	222	gcC*g	C → T	Pro → Leu	50
			223	gCct	—C	Pro → ... stop	70
YK 124B	knuckle	+	331	ttCa	C → T	Gln → stop	60

Bold, sample with more than one p53 mutation; boxed, patient with p53 mutation in more than one actinic keratosis; n.i., non-informative at codon 72; *, potential 5-methylcytosine; —T indicates T deleted; mutation signal intensity represents sequencing-gel band intensity of mutant divided by intensity of (wild type + mutant). AKs were removed by Mohs surgery. For samples YK101–118, tissue was also removed from adjacent sun-exposed skin more than 1 cm away from the AK, and from sun-shielded skin on the inner aspect of the arm. Half of each specimen was fixed in neutral-buffered formalin, embedded in paraffin and stained with haematoxylin-eosin. The remainder was frozen and DNA isolated using proteinase K. Some paraffin sections were microdissected before DNA isolation to reduce contamination with normal tissue⁴. The absence of associated SCC in these patients excluded the possibility that the lesion studied was a morphological variant of SCC. Exons 2 to 11 of the p53 gene were amplified by PCR and directly sequenced using the buffers, primers, cycling conditions and controls described previously^{4,5}. To determine allelic loss, the region of polymorphic codon 72 was amplified from microdissected paraffin sections and PCR fragments were restricted. p53 protein was detected in unbaked paraffin sections using rabbit polyclonal antibody CM-1 (NovoCastra, Newcastle, UK) at a dilution of 1:4,000 (ref. 5). Staining was restricted to the epidermis and was absent when CM-1 antibody was omitted.

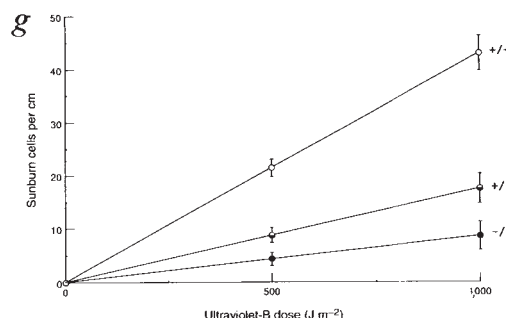
FIG. 1 Sunburn cell formation in different *p53* genotypes. a, b, Haematoxylin–eosin staining of epidermis of *p53*^{+/+} mice, 0 and 500 J m⁻² UV-B; c, d, *p53*^{-/-}, 0 and 500 J m⁻². e, f, FISEL detection of nuclear DNA strand breaks in *p53*^{+/+} mice after 0 and 500 J m⁻². g, Dose response of sunburn-cell formation in different *p53* genotypes.

METHODS. The germline *p53* gene was disrupted between exons 2 and 6, preventing production of *p53* protein⁸; mice were C57BL6 × 129/sv hybrids, age 7–8 weeks. Hair was removed by waxing a 2-cm² region of the back; because removal of the stratum corneum stimulates cell proliferation, this procedure also enhances the number of sunburn cells seen. 12 h later mice were irradiated 8 cm beneath three UV-B lights (FS20T12-UVB; National Biological) at a dose rate of 11.3 J m⁻² s⁻¹; vertical movement was restrained by a wire screen with 1.2-cm grid. Mice were killed 24 h after UV-B, skin flaps were fixed and embedded, and 5-μm sections stained with haematoxylin–eosin. To detect DNA strand breaks, the TUNEL method³⁰ was modified so that terminal transferase directly incorporated fluorochrome-labelled dCTP (Cy-5; Biological Detection Systems, Pittsburgh, USA). Cells were visualized with a BioRad MRC-600 laser confocal microscope, with RHS filter set. Criteria for scoring sunburn cells were intensely eosinophilic cytoplasm, pycnotic nuclei, and separation from adjacent cells. For each of 2–5 mice per point, about 6 haematoxylin–eosin sections, spaced three sections apart, were counted for sunburn cells per linear cm of epidermis. Data for each genotype were analysed using weighted linear regression of sunburn cell frequency versus UV dose, with weights proportional to the mean at each dose. Bars represent standard errors; slope differences were statistically significant by *t*-test ($P < 10^{-6}$ for *+/+* versus *-/-*; $P = 8 \times 10^{-6}$ for *+/+* versus *+/-*; $P = 0.03$ for *+/-* versus *-/-*). Sunburn cells: 400 ×, tungsten bulb, LBD-2 standard daylight blue filter, Kodak Ektar 25 film; FISEL: 504 ×, Kodak Elite 100 film, 1 s.



and now they are shown to depend largely on a gene, *p53*, that prevents tumours in mouse skin¹⁶ and is mutated in most human skin cancers and precancers.

If the *p53* amino-acid substitutions found in AK are as detrimental to sunburn-cell formation as the null mutations studied here, chronic sunlight exposure would lead to a sequence of events resembling the early stages of human skin cancer (Fig. 2). In particular, sunburn subsequent to a *p53* mutation will act as a selection pressure favouring the cell containing that mutation. Ultraviolet-damaged normal cells will die as sunburn cells, but about half of damaged *p53*^{+/+} cells will be resistant to apoptosis (Fig. 1g). These mutated cells can then clonally expand into an actinic keratosis¹⁷. Because the differential selection must occur after the *p53* mutation, this second effect of sunlight fits the definition of tumour promotion¹⁸. Ultraviolet light can act as a tumour promoter in mouse skin, as can other DNA-damaging agents such as oxygen radicals^{19,20}. The influence of selection can be profound owing to the exponential nature of cell proliferation: growth of mouse skin papillomas can be sustained by only a 5% excess of cell production over cell loss²¹. The expanding cell-death-defective clone: (1) presents a larger target for future ultraviolet exposure (refs 17, 18 and refs therein), and (2) is a target in which a greater fraction of cells can survive irradiation with ultraviolet to carry an additional mutation in *p53* or another gene¹⁷. These effects can increase the number of mutant cells without requiring a mutator phenotype. The delicate balance between cell death and cell proliferation rates can allow



even a two-gene carcinogenesis mechanism to proceed slowly enough to appear multigenic²². The biological effects of *p53* mutations on keratinocytes will be quantitative rather than all-or-none.

The mutagenicity of sunlight in the *p53* gene and the subsequent selection pressure exerted by sunburn cell formation, acting over the course of years, can account for the salient features of human skin carcinogenesis: (1) skin cancer and the precancerous actinic keratosis are most frequent in individuals who sunburn rather than tan, particularly those of Celtic descent^{23,24}; (2) an actinic keratosis usually regresses in the absence of exposure to sun but progressively enlarges if exposure

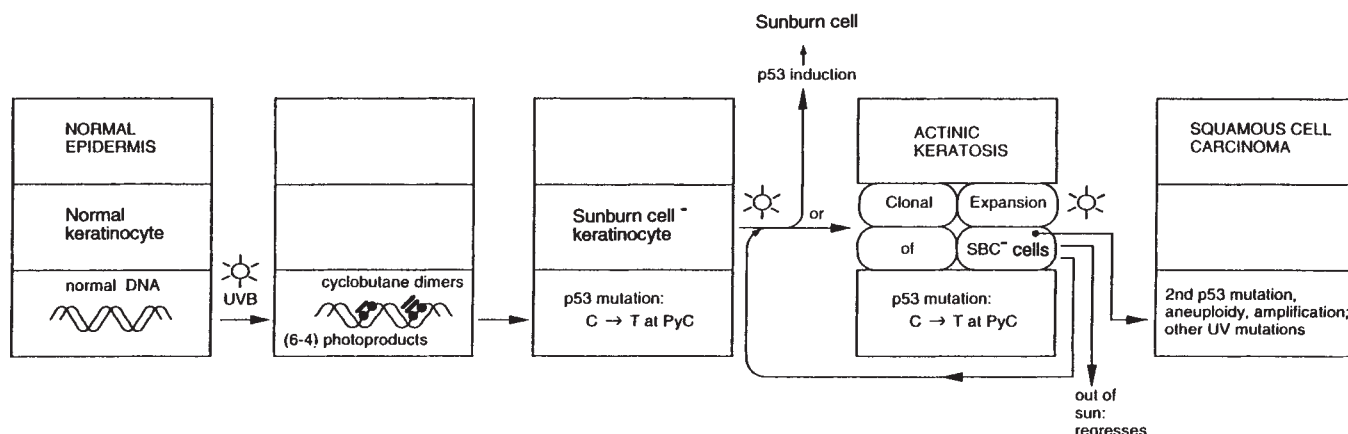


FIG. 2 Early genetic, cellular and tissue-level events in human skin cancer: selection for p53-mutated cells by repeated sunburn. Sunlight UV-B induces cyclobutane pyrimidine dimers and (6-4) photoproducts in epidermal cell DNA; most photoproducts are repaired, but upon DNA replication some cells acquire a C → T point mutation in the p53 gene (refs 4, 5) and Table 1). At the cellular level, these p53^{+/−} keratinocytes (sunburn cell[−] keratinocytes; SBC[−] cells) are partially defective for sunburn cell production (Fig. 1g). When the next sunlight exposure induces p53 protein¹³, normal cells UV-damaged during S phase die by apoptosis, but only about half of the p53^{+/−} cells die (Fig. 1g). A p53^{+/−} cell can then clonally expand, and may become an actinic keratosis (Table 1). Thus, sunburn subsequent to a p53 mutation can act as a

selection pressure for the mutated, damage-resistant, cell. These events may be frequent, because the lifetime-expectancy of AK in Australia approaches 100% (ref. 26). Other mutated genes may contribute to the AK phenotype. In the absence of further sun exposure, 25% of actinic keratoses regress each year²⁵. But continued sun exposure selects for further clonal expansion of the SBC[−] cells in the AK, potentially allowing one cell to become p53^{−/−} (ref. 5 and Table 1; YK121A and 124B may contain point-mutant subclones emerging within an allelic-loss AK). Homozygous mutation could lead to aneuploidy and gene amplification¹², to UV-induced mutations in additional genes, and to squamous cell carcinoma of the skin⁴.

continues²⁵; (3) skin cancers and precancers can acquire sunlight-induced p53 mutations at a rate much higher than is achievable *in vitro*⁵; (4) although sunlight exposure significant for actinic keratosis and squamous cell carcinoma occurs in childhood, skin cancer appears after forty or more years of chronic relatively low-dose sun exposure^{24,26}.

The clinical consequence of early mutation induction by sunlight contrasts with tissues in which p53 influences conversion of low-grade tumours to more malignant tumours²⁷. SCC, already more frequent in Australia and the United States than colon, lung or breast cancer, has been increasing exponentially²⁸, but

these rates are based on the elderly population in whom SCC arise. Because sunlight acts early, these rates should increase further as individuals with greater early-sun exposure, now in their 30s and 40s, reach the age at which tumours appear. □

TABLE 2 Analysis of sun-damaged skin flanking an actinic keratosis for the p53 mutation found in that actinic keratosis

Sample	Codon	Mutation frequency
YK 107B	84	10 ^{−3}
YK 110E	331	<10 ^{−3}
YK 114B	178	<10 ^{−3}
YK 114B	342	10 ^{−3}
YK 115D	241	<10 ^{−3}
YK 116D	282	10 ^{−2}
YK 117C	179	<10 ^{−3}
YK 118D	279	10 ^{−3}

For 8 cases in which a p53 mutation was found in an AK and a biopsy of sun-damaged skin had been taken, 1–3 cm distant to ensure that only non-AK tissue was examined, DNA was isolated from the sun-damaged skin and amplified by PCR. To detect low frequencies of the actinic keratosis mutation, 28-nucleotide primers were extended opposite the mutated base (T) with [³²P]dATP (Amersham) as described²⁹, except that the reaction included *Pfu* polymerase (Stratagene), 50 μM dideoxyGTP (New England Biolabs) to reduce nonspecific incorporation, and, depending on the sequence, either 3 μM dNTP, corresponding to the 3' base of the primer, or a primer with phosphorothioate linkages at the 3' two positions to reduce primer shortening by the proof-reading exonuclease of *Pfu*. A similar reaction with ³²P-dGTP measured the frequency of wild-type molecules to correct for variations in DNA concentration and loading. Template was 10 ng of PCR product. Mutation frequency was determined by comparing the signal strength of the skin sample to a standard curve.

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Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM

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SYNAPTIC membranes express cell adhesion molecules¹. Here we investigate the role of the neural cell adhesion molecules L1 and NCAM in hippocampal long-term potentiation (LTP), a sustained-use-dependent increase in synaptic efficacy that has been implicated in learning and memory². L1 and NCAM mediate cell interactions during neural development³ and are strongly expressed in the hippocampus^{4,5}. They cooperate to strengthen L1-dependent cell adhesion⁶⁻⁹ and are coupled to second messenger pathways¹⁰⁻¹². We show that LTP in CA1 neurons of rat hippocampal slices was reduced by application of various L1 and NCAM antibodies, recombinant L1 fragments, and upon dissociation of the L1/NCAM complex through oligomannosidic carbohydrates and NCAM peptides. Neither the activation of NMDA (*N*-methyl-D-aspartate) receptors nor the maintenance of LTP was affected. These results suggest that L1 and NCAM modulate the development or the stabilization of LTP¹³.

LTP of the field excitatory postsynaptic potential (e.p.s.p.) in the CA1 stratum pyramidale was induced by θ -burst stimulation (TBS)¹⁴. Local ejection of polyclonal antibodies against L1 within the apical dendritic field of CA1 neurons produced a concentration-dependent decay of LTP (Fig. 1*b, c*). At the highest concentration tested (6.2 mg ml⁻¹), the field e.p.s.p. slope returned to the baseline level within 30 min after TBS (100 $\pm$ 3%; n = 5; P < 0.001; indicated values of LTP are taken 50 min after TBS as percentage of pre-TBS baseline). In the same slices, LTP simultaneously recorded at a control site apart from the ejection site was found to be 163 $\pm$ 8% (n = 25; Fig. 1*a, c*). A polyclonal antibody against the recombinant immunoglobulin-like domains I–VI of L1 induced a similar reduction of LTP (114 $\pm$ 11%; n = 5; P < 0.01; control site: 161 $\pm$ 5%; n = 5; not shown). Specificity of the action of the L1 antibodies on LTP was assessed by applying (1) a non-immune rabbit serum; (2) an IgG fraction derived from non-immune rabbit serum; and (3) an IgG fraction of a polyclonal antibody raised against mouse liver cell membranes that effectively binds to neuronal and glial cell surfaces in rat hippocampal slices¹⁵. These antibodies did not interfere with LTP (162 $\pm$ 7%, 161 $\pm$ 7% and 166 $\pm$ 10%, respectively; n = 5; data not shown and Fig. 1*c*). Interestingly, continuous application of the L1 antibodies for 60 min without TBS induced no

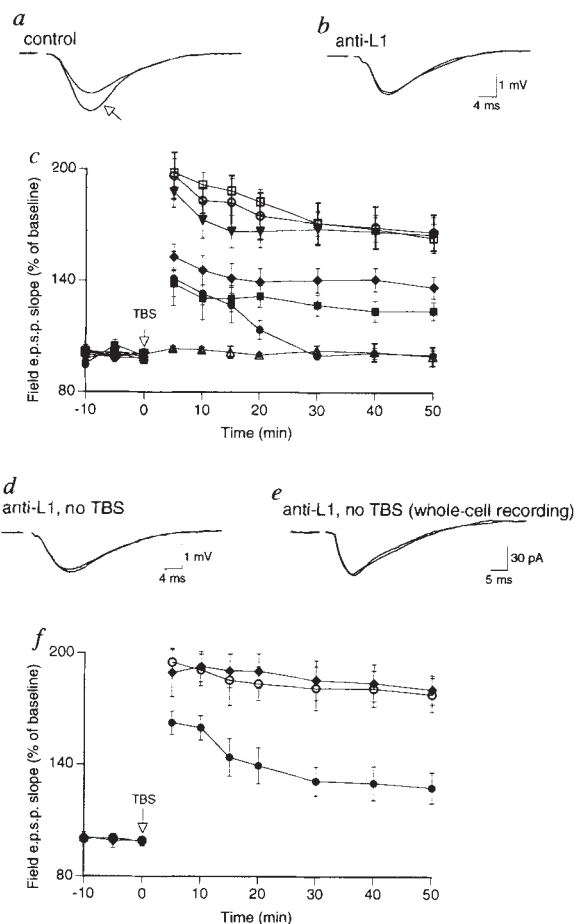


FIG. 1 Effect of L1 antibodies and L1 fragments on LTP in CA1. *a*, Field e.p.s.p.s recorded before and 50 min after (arrow) TBS at the control site. *b*, Field e.p.s.p.s recorded before and 50 min after TBS in the presence of L1 antibodies (IgG fraction)²⁴. *c*, Time course of LTP (field e.p.s.p. initial slope) in the presence of L1 antibodies at increasing concentrations (0.06 mg ml⁻¹, ▽; 0.6 mg ml⁻¹, ◆; 2 mg ml⁻¹, ■; 6.2 mg ml⁻¹, ●) with the following controls: (1) LTP recorded from a control site in the same slices (□); (2) polyclonal antibodies against mouse liver membranes¹⁵ (IgG fraction, 3.5 mg ml⁻¹; ○); and (3) L1 antibodies without induction of LTP (6.2 mg ml⁻¹, △). *d*, Field e.p.s.p.s recorded before and 60 min

after the application of L1 antibodies in the absence of TBS. *e*, Intracellular excitatory postsynaptic currents (holding potential: -70 mV) recorded before and 30 min after the application of L1 antibodies in the absence of TBS. *f*, Time course of LTP (field e.p.s.p. initial slope) in the presence of the immunoglobulin-like domains I–VI (216 µg ml⁻¹; 20 mM Tris-HCl, pH 7.6; ●) and of the fibronectin type III homologous repeats I–V (225 µg ml⁻¹; 20 mM Tris-HCl, pH 7.6; ◆) of L1, compared to medium (20 mM Tris-HCl, pH 7.6; ○).

METHODS. Rat hippocampal slices (400 µm) were maintained in an interface chamber and perfused at 35 °C with a medium containing (mM): NaCl, 124.0; KCl, 2.5; MgSO₄, 2.0; CaCl₂, 2.5; KH₂PO₄, 1.25; NaHCO₃, 26.0; glucose, 10; sucrose, 4; bubbled with 95% O₂/5% CO₂ (pH 7.4). The CA1 stratum radiatum was stimulated (0.05 Hz, 100 µs) at a stimulus strength adjusted to evoke field e.p.s.p.s equal to 30% of their maximum amplitudes without superimposed population spike. Absolute baseline values of the field e.p.s.p.s nonsignificantly varied between 0.205 and 0.259 mV ms⁻¹, and between 0.795 and 0.863 mV ms⁻¹ for the experiments using the oligomannosidic carbohydrates. Field e.p.s.p.s were recorded from the CA1 stratum radiatum by means of two micropipettes (2M NaCl, 1–5 MΩ) positioned about 300 µm apart from the stimulation electrode on each side. Field potentials are depicted as averages of 4 consecutive signals. After stable recording for at least 15 min, antibodies or protein fragments (in 20 mM PBS, pH 7.4) were ejected onto the CA1 dendritic field in the vicinity (50–75 µm) of one of the two recording electrodes (the one adjusted at 30%, the other being about 30% of maximum) using a microinjection pipette delivering 5 nl every 10 s throughout the experiment. 20 min later, LTP was induced using a TBS paradigm¹⁵ consisting of three stimulus trains spaced by 4 s. Duration of the stimulation pulses was doubled during TBS. Results are expressed as means $\pm$ s.e.m. of the field e.p.s.p. initial slope in per cent of the baseline values recorded during the 10 min before TBS for 5 to 6 slices from at least 3 animals. Statistical evaluations were performed by analysis of variance (against the control reagents) on the last 20 min of the experiments to exclude early phenomena, like post-tetanic potentiation. Whole-cell recordings²⁵ were obtained at 30 °C using electrodes (3–8 MΩ) filled with a solution containing (in mM): potassium gluconate, 129; KCl, 5; MgCl₂, 1; CaCl₂, 1; HEPES, 5; BAPTA, 5; Na-ATP, 10 and Na-GTP, 0.3, pH 7.3.

change in the field e.p.s.p. (Fig. 1d; $n=5$). Similarly, in intracellular recordings, L1 antibodies did not modify the shape of the action potential, the slope and amplitude of the evoked post-synaptic currents (Fig. 1e; $n=6$), the resting membrane potential (-62.0 ± 2.9 mV versus -63.9 ± 2.8 mV after 30 min of L1 antibody application; $n=6$), or the cellular input resistance (93.4 ± 22.5 M Ω and 97.9 ± 17.1 M Ω for the antibodies against L1 and liver membranes, respectively; $n=6$).

As L1 is a homophilic cell adhesion molecule^{16,17}, we accordingly used recombinantly expressed L1 fragments as ligands for the L1 molecules on CA1 pyramidal neurons. Application of a fragment comprising the immunoglobulin-like domains I–VI induced a reduction in LTP ($216 \mu\text{g ml}^{-1}$; $128 \pm 8\%$; $n=5$, $P<0.01$; Fig. 1f), whereas the fragment containing the fibronectin type III homologous repeats I–V was not active ($225 \mu\text{g ml}^{-1}$; $n=5$; Fig. 1f). These results endorse the idea that

L1 is involved in the generation of LTP, and emphasize the role of its immunoglobulin-like domains.

As homophilic binding of L1 is strengthened by lateral interaction of the L1 molecule with NCAM^{7,8}, the involvement in LTP of NCAM itself and of its interaction with L1 were assessed. Affinity-purified polyclonal antibodies against NCAM reduced LTP ($128 \pm 7\%$; $n=5$, $P<0.01$; Fig. 2a) when compared to control antibodies (anti-liver membranes, $165 \pm 6\%$; $n=5$). The LTP recorded at the control site was $166 \pm 8\%$ ($n=10$). Another polyclonal NCAM antibody reduced LTP as well ($130 \pm 8\%$; $n=5$, $P \leq 0.06$; control site: $176 \pm 7\%$; $n=5$; result not shown). Like the L1 antibodies, the antibodies against NCAM had no effect on basal synaptic transmission during 60 min of application (Fig. 2a; $n=5$). More precisely, the interaction of L1 with NCAM depends on oligomannosidic carbohydrates, carried by L1, which bind to the fourth immunoglobulin-like domain of

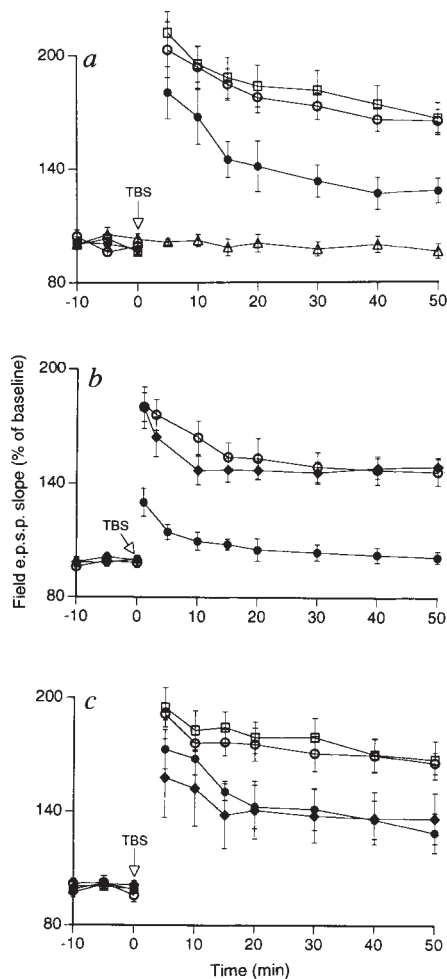


FIG. 2 Effect of NCAM antibodies, oligomannosidic carbohydrates and NCAM-peptides on LTP. **a**, Time course of LTP in the presence of affinity-purified NCAM antibodies ($50 \mu\text{g ml}^{-1}$, ●), with the following controls: (1) LTP recorded from a control site in the same slices (□), (2) Polyclonal antibodies against mouse liver membranes ($50 \mu\text{g ml}^{-1}$, ○), and (3) NCAM antibodies (Δ) without induction of LTP. **b**, Time course of LTP in the presence of oligomannosidic carbohydrates (●), control carbohydrates (○) derived from asialofetuin (both $100 \mu\text{M}$ in the perfusion medium), and in the absence of carbohydrates (○). LTPs in **a**, **c** and **b** were recorded in separate laboratories; this explains the different magnitudes in control LTP. **c**, Time course of LTP in the presence of the peptides MS1 (○, 2 mg ml^{-1}), MS2 (●, 2 mg ml^{-1}) and MS1–10 (▲, 2 mg ml^{-1}) and of LTP recorded from a control site in the same slices (□). The peptide MS1 contains the first 19 amino acids of the first Ig-like domain, peptide MS2 the first 19 and peptide MS1–10 the first 10 amino acids of the fourth Ig-like domain of NCAM, respectively⁸.

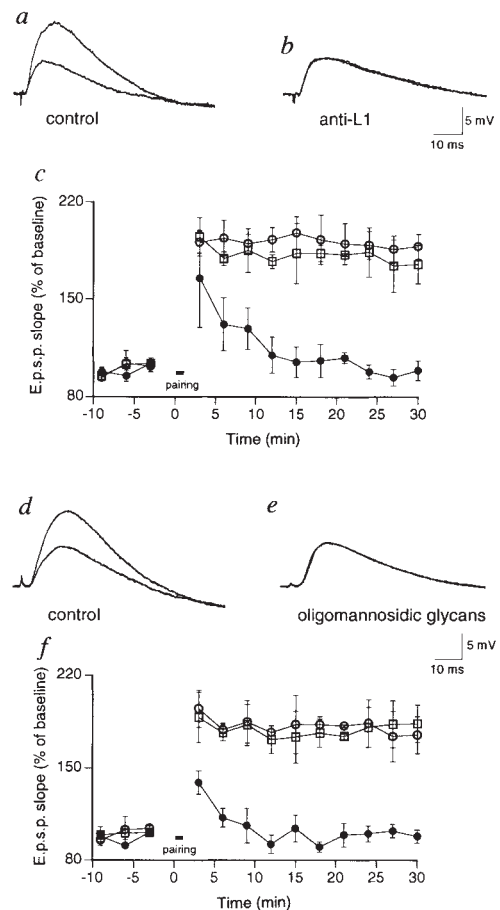


FIG. 3 Effect of L1 antibodies and oligomannosidic carbohydrates on pairing-induced LTP. **a**, Intracellular e.p.s.p.s recorded before and 30 min after pairing in the presence of control antibodies (anti-liver membranes). **b**, Intracellular e.p.s.p.s recorded before and 30 min after pairing in the presence of L1 antibodies. **c**, Time course of intracellular LTP in the presence of L1 antibodies (6.2 mg ml^{-1} ; ●) and control antibodies (anti-liver membranes, 3.5 mg ml^{-1} ; ○). **d**, Intracellular e.p.s.p.s recorded before and 30 min after pairing under control conditions. **e**, Intracellular e.p.s.p.s recorded before and 30 min after pairing in the presence of oligomannosidic carbohydrates. **f**, Time course of intracellular LTP in the presence of oligomannosidic carbohydrates ($100 \mu\text{M}$; ●) and under control conditions (○). **METHODS.** Patch electrodes were filled with a solution containing (in mM): potassium gluconate, 135; NaCl, 8; HEPES, 10; EGTA, 0.2; Mg-ATP, 2 and Na-GTP, 0.2, pH 7.25. The extracellular medium contained $3 \mu\text{M}$ picrotoxin and 1.3 mM MgSO_4 . Input resistance was constantly monitored and cells showing more than 10% change were excluded from the analysis. After acquisition of baseline responses (half maximal e.p.s.p.), LTP was induced by pairing a 2 Hz stimulation (same stimulus strength as baseline) for 60s with a depolarization to -10 mV.

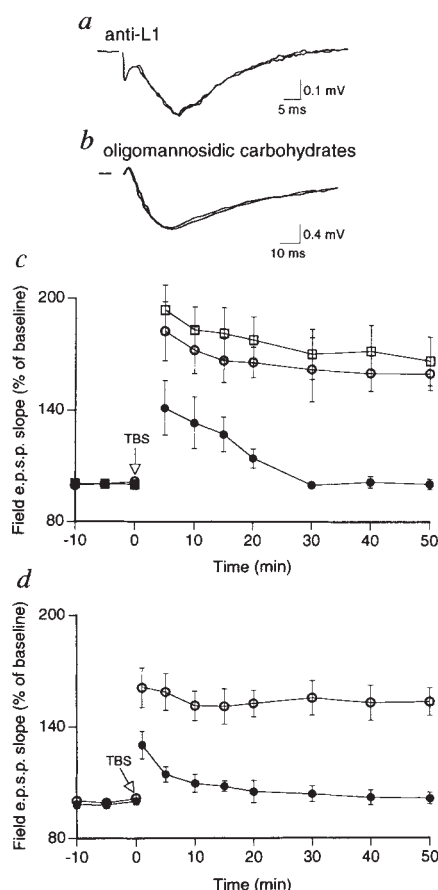


FIG. 4 Effect of L1 antibodies and oligomannosidic carbohydrates on NMDA receptor-mediated synaptic transmission and on previously established LTP. *a*, NMDA receptor-dependent field e.p.s.ps recorded in the presence of CNQX (30 μ M) before and after 30 min application of L1 antibodies. *b*, NMDA receptor-dependent field e.p.s.ps recorded in the presence of CNQX (10 μ M) before and after 60 min application of oligomannosidic carbohydrates. *c*, Time course of LTP in the presence of L1 antibodies applied either throughout the experiment (6.2 mg ml⁻¹; ●, same data as in Fig. 1c) or beginning 10 min after TBS (6.2 mg ml⁻¹; ○) and of LTP recorded from a control site in the same slices (□). *d*, Time course of LTP in the presence of oligomannosidic carbohydrates applied either throughout the experiment (100 μ M; ●, same data as in Fig. 2b) or starting 10 min after TBS (100 μ M; ○).

METHODS. NMDA receptor-mediated field e.p.s.ps were isolated by adding CNQX to the perfusion medium 20 min before application of antibodies or carbohydrates. At the end of each experiment, it was verified that D(-)-2-amino-5-phosphonopentanoic acid (D-AP5, 30 μ M) completely suppressed these responses.

NCAM⁸. This interaction is abolished by soluble oligomannosides and by synthetic peptides comprising part of the lectin sites of the fourth immunoglobulin-like domain of NCAM⁸. We observed that bath application of oligomannosidic carbohydrates (100 μ M) strongly reduced LTP ($102 \pm 5\%$; $n=6$, $P<0.001$; Fig. 2b), whereas the control carbohydrates derived from asialofetuin had no effect ($n=6$). Local ejection of two synthetic peptides from the fourth immunoglobulin-like domain of NCAM also reduced LTP ($129 \pm 10\%$; 2 mg ml⁻¹, $n=5$, $P<0.05$, for peptide MS1-10 and $137 \pm 13\%$; 2 mg ml⁻¹, $n=5$, $P=0.07$, for peptide MS2; Fig. 2c), whereas the peptide from the first immunoglobulin-like domain remained without effect (peptide MS1; $n=5$; 2 mg ml⁻¹). The LTP recorded at the control site was $167 \pm 10\%$ ($n=15$). These data indicate that a stable L1/NCAM complex might be a prerequisite for a full development of LTP.

The decreases in LTP we recorded did not result from an interference of the various active compounds with the tetanic

stimulus, with the depolarization-dependent activation of NMDA receptors, or with the maintenance phase of LTP, for the following reasons: LTP induced in single cells by low-frequency stimulation paired with postsynaptic depolarization was blocked by both the L1 antibodies ($99 \pm 7\%$, 30 min after pairing; $n=5$, $P<0.001$; Fig. 3b, c) and the oligomannosidic carbohydrates ($99 \pm 6\%$; $n=4$, $P<0.001$; Fig. 3e, f) when compared to control ($166 \pm 15\%$; $n=4$; Fig. 3c, d, f), to anti-liver membrane antibodies ($188 \pm 9\%$; $n=5$; Fig. 3a) and to control carbohydrates ($184 \pm 14\%$; $n=2$; Fig. 3f). Then, as shown in Fig. 4a and b, NMDA-receptor-mediated synaptic responses recorded in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 and 30 μ M, respectively; $n=5$) were not modified by L1 antibodies and oligomannosidic carbohydrates. Finally, application of the L1 antibodies and of the oligomannosidic carbohydrates 10 min after TBS ($n=5$, $P<0.001$; Fig. 4c, d) did not modify previously established LTP. These data suggest a requirement of L1 or of the L1/NCAM complex within the development or stabilization of LTP rather than within the induction or maintenance¹³.

The mechanisms by which the L1 and NCAM antibodies, the recombinant L1 fragments or the dissociation of the L1/NCAM complex eventually influence LTP may result from their ability to interfere with associated changes in synaptic morphology^{18,19}. Alternatively, dissociation of the L1/NCAM complex and binding of antibodies or specific fragments may trigger or attenuate signal transduction mechanisms¹⁰⁻¹², resulting in a decreased synaptic plasticity without affecting basal synaptic transmission. In either possibility, a functional L1/NCAM complex appears to be necessary for proper development of LTP; the potential plasticity of a synapse could then be seen as depending on the relative local concentrations of L1 and NCAM, and on their interactions with other synaptic adhesion molecules. Furthermore, intracerebral injection of L1 and/or NCAM antibodies has been reported to impair learning and retrieval tasks²⁰⁻²² (A. B. Scholey *et al.*, manuscript in preparation; S. Arami *et al.*, manuscript in preparation), and inactivation of the NCAM gene in transgenic mice resulted in spatial learning deficits similar to those obtained with NCAM antibody injection in rats²³. Thus, taken together, these results implicate cell adhesion molecules of the immunoglobulin superfamily in synaptic plasticity. □

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Control of type-D GABAergic neuron differentiation by *C. elegans* UNC-30 homeodomain protein

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THE *Caenorhabditis elegans* gene *unc-30* is required for the development and functioning of the 19 inhibitory GABAergic (γ -aminobutyric-acid-secreting) type D motor neurons, which control locomotion¹⁻⁴. In *unc-30* mutants the D neurons lack GABA² and have defects in axonal pathfinding and synaptic connections (J. White, personal communication). We report here that *unc-30* encodes a homeodomain protein that is present in the nuclei of the D neurons at high levels in young larvae, in which the motor circuitry is formed, and at low levels in older animals. The UNC-30 protein is also present in six non-GABAergic neurons and is absent from the seven non-D-type GABAergic neurons. Ectopic expression of *unc-30* induced GABA expression in cells that are normally not GABAergic. We propose that *unc-30* functions as a transcriptional regulator within the type D neurons to control their terminal differentiation and that *unc-30* is sufficient in some but not all cell types to induce GABA expression.

We cloned the *unc-30* gene by using strain-specific restriction fragment length polymorphisms (RFLPs) to position *unc-30* on the physical map of the *C. elegans* genome^{5,6} and then identifying DNA clones that rescued the *Unc-30* mutant phenotype in transgenic animals^{7,8} (Fig. 1a; also see Fig. 1 legend). The *unc-30*-rescuing activity was localized to a 6.3 kilobase (kb) *EcoRI*-*Clal* genomic fragment (Fig. 1b). We also found that two *unc-30* mutants, *unc-30(e926)* and *unc-30(e2327)*, both of which were isolated after mutagenesis with ³²P-phosphate^{9,10}, had deletions within this genomic fragment (Fig. 1c, d).

Using the 6.3 kb fragment as a probe for a northern blot, we detected a single transcript of 1.3 kb in mixed-stage poly(A)⁺ RNA (data not shown). We isolated four complementary DNA clones from a cDNA library prepared from mixed-stage poly(A)⁺ RNA. Each of these clones had an insert of about 1.3 kb. We determined the complete sequence of one cDNA clone, c16. This clone has an insert of 1,257 nucleotides and encodes a single open reading frame of 318 amino-acid residues containing a homeodomain from residue 111 to 170 (Fig. 2a). We sequenced genomic DNAs corresponding to the homeodomain region in the 11 *unc-30* mutants besides *unc-30(e926)* and *unc-30(e2327)* (see Fig. 2 legend) and found that two carry nucleotide changes in the homeodomain (Fig. 2b): *unc-30(e165)* encodes glutamic acid instead of valine at homeodomain position 45 in helix 3, and *unc-30(e646)* encodes an ochre stop codon instead of arginine at position 44. The UNC-30 homeodomain is a divergent member of the homeodomain family, sharing 58% amino-acid identity with its closest relatives, *Drosophila melanogaster* Otd¹¹ and murine Pax-3¹². At position 50 of the homeodomain, a site that contributes to the determination of DNA-binding specificity¹³, the UNC-30 protein has a lysine, as is also found in the *Drosophila* Bcd homeodomain¹⁴. We determined the sequence of the 6.3 kb *EcoRI*-*Clal* genomic fragment and found that this fragment does not contain the presumptive last exon, which encodes the carboxy-terminal 43 amino acids (Fig. 2a), suggesting that these 43 amino acids are not essential for UNC-30 function. Because there are several in-frame stop codons 5' to the putative translational initiation methionine, and

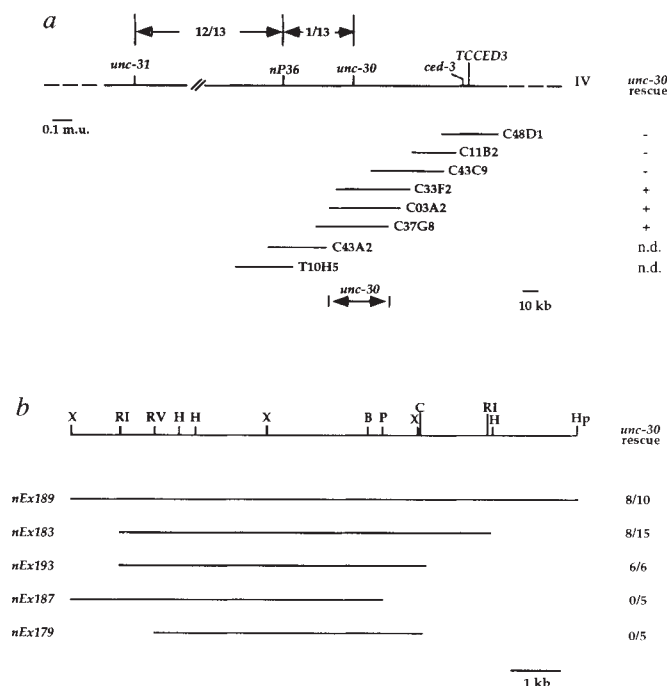


FIG. 1 a, A genetic map of part of the right arm of chromosome IV is shown above, and cosmid clones from a part of the physical map of this region are shown below. Our RFLP mapping placed *unc-30* between *nP36* and *TCCED3*. Germ-line transformation identified *unc-30*-rescuing activity within the overlapping regions of the three cosmid clones *C33F2*, *C03A2* and *C37G8*. Plus, Cosmid clone restored the normal locomotory pattern to *unc-30(e191)* animals; minus, cosmid clone did not restore normal movement. These results localized the *unc-30*-rescuing activity to a 25 kb region. n.d., Not determined; m.u., map units. b, Restriction map for subclones tested for *unc-30* rescuing activity. Stable transgenic lines were established and examined for both the locomotory pattern of the animals and the presence of GABA in the D neurons. The fraction on the right indicates the number of rescued lines among the total number of stably maintained transgenic lines. The shortest fragment that contained *unc-30*-rescuing activity is the 6.3 kb *EcoRI*-*Clal* fragment carried in extrachromosomal array *nEx193*. Restriction enzyme abbreviations are: RI, *EcoRI*; RV, *EcoRV*; X, *XbaI*; B, *BamHI*; H, *HindIII*; C, *Clal*; P, *PstI*; Hp, *HpaI*. c, Southern blot showing effects of deletions in the *unc-30* mutants *e926* and *e2327*. A Southern blot was prepared from wild-type (N2) and *unc-30* mutant DNAs digested with *XbaI*, *BamHI* and *HindIII* and was probed with the ³²P-labelled 4.5 kb *EcoRV*-*BamHI* fragment. N2, The wild-type Bristol strain; *unc-30(e926)*; *unc-30(e2327)*. d, Diagram of the *unc-30* region. The restriction map of the *unc-30* locus determined from the DNA sequence of the *EcoRI*-*Clal* fragment and the c16 cDNA (see Fig. 2) is presented above. Restriction enzyme abbreviations are as in b. Boxes, exons; black boxes, homeodomain (Fig. 2). The region deleted in *unc-30(e2327)* is represented by a solid line. Dashed lines indicate the regions within which breakpoints of *unc-30(e926)* and *unc-30(e2327)* reside. Using the 6.3 kb *EcoRI*-*Clal*, 2.4 kb *EcoRV*-*XbaI*, 2.1 kb *XbaI*-*BamHI* and 1.0 kb *BamHI*-*XbaI* fragments as probes on genomic Southern blots prepared from *HindIII*-*XbaI* and *HindIII*-*BamHI* digests, we detected no abnormality in regions outside the 1.6 kb *HindIII*-*XbaI* fragment in *unc-30(e926)* DNA and an absence of all detected DNA fragments left of the *BamHI* site in *unc-30(e2327)* DNA (data not shown). Thus, *unc-30(e926)* has a 0.1 kb deletion within the 1.6 kb *HindIII*-*XbaI* fragment, and *unc-30(e2327)* has a large deletion that breaks within the 2.1 kb *XbaI*-*BamHI* fragment.

METHODS. a, Genetic mapping placed *unc-30* between *unc-31* and *ced-3* on chromosome IV^{17,18}. We localized *unc-30* between two Tc1 Bristol-Bergerac strain dimorphisms *TCCED3* and *nP36*¹⁸ by constructing heterozygotes containing a Bristol strain N2-derived chromosome *unc-31(e928) unc-30(e191) IV* in *trans* to a wild-type Bergerac strain B0 chromosome^{6,19}. One of 13 *Unc-30* non-*Unc-31* recombinants carried the *nP36* dimorphism on Southern blot analysis, which placed the *unc-30* gene between *nP36* and *TCCED3*. Cosmid clones from this region were injected into the oocytes of *unc-30(e191)* animals⁷, and if F1 progeny showed wild-type locomotion they were scored as rescued; ►

► some rescued F1 produced non-Unc progeny in the following generations, indicating that the transgene was maintained through the germ-line. *b*, To assay cosmid subclones, we coinjected into the syncytial gonad both the test DNA and the dominant roller marker plasmid pRF4⁸. Roller lines containing both the test DNA and pRF4 in extrachromosomal arrays were established and scored for animals with wild-type locomotion. These roller lines were examined further by staining with anti-GABA antisera²⁰. In the behaviourally rescued animals, the D neurons expressed GABA, and their processes as revealed by anti-GABA staining appeared to have normal morphologies. *c*, Worm genomic DNA was isolated as described²¹, and Southern blots were obtained according to standard procedures.

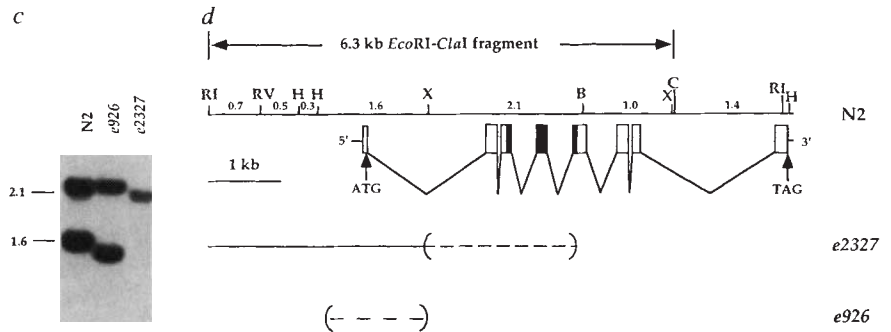


FIG. 2 *a*, Nucleotide and predicted amino-acid sequences (single-letter code) corresponding to the c16 *unc-30* cDNA. Nucleotides are numbered on the right beginning with the first nucleotide present in the cDNA. Amino acids are numbered in italics on the right beginning with the first predicted methionine. The positions of splice sites were determined by comparison of the cDNA and the genomic sequences (not shown) and are marked by arrowheads. Amino acids that are not encoded within the 6.3 kb *EcoRI-ClaI* rescuing fragment (see text) are underlined. The homeodomain is marked by bold letters. *b*, Alignment of the UNC-30 homeodomain with other homeodomains. The fractions of identities between UNC-30 and other proteins are listed to the right. Asterisks indicate the positions most conserved among all homeodomains²². Amino-acid residues at homeodomain position 50, which is a determinant of DNA-binding specificity¹³, are italicized and in bold face. Horizontal lines mark the three homeodomain α -helices H-1, H-2 and H-3, based on X-ray crystallographic studies²³. Homeodomain sequences correspond to those of the *Drosophila* Otd¹¹, *Drosophila* Prd¹⁴, *Drosophila* Antp²⁴, *Drosophila* Bcd¹⁴, *Xenopus laevis* Gsd²⁵, murine Pax-3¹², murine Otx1²⁶ and *C. elegans* UNC-4²⁷ proteins.

METHODS. The 6.3 kb genomic fragment that contained *unc-30*-rescuing activity was used to screen 500,000 plaques of a λ ZAP cDNA library prepared from mixed-stage poly(A)⁺ RNA²⁸ (a gift from R. Barstead and R. Waterston). Four positive clones were identified, all of which had the same size insert and shared sequences at their ends and therefore appeared to be derived from the same clone. We determined the complete DNA sequences of both strands of one isolate, c16, using Sequenase 20 (USB), essentially according to the instructions provided by the manufacturer. The sequence of the 6.3 kb *EcoRI-ClaI* genomic fragment was determined similarly. Genomic DNAs from strains carrying each of 11 *unc-30* alleles (e165, e191, e318, e596, e646, e727, e1169, e2384, n2389, n2390 and n2391) were amplified by asymmetrical PCR²⁹ using primers flanking the homeodomain. The primers used were: 5'-TGAAAGTACG-AATGGAGGG-3' and 5'-CGTGGTGCCCGTCCGTTGTC-3'. We determined the DNA sequences of both strands of the genomic sequence corresponding to the homeodomain region. The mutant e165 has an AT-to-TA transversion, converting a valine to a glutamic acid. The mutant e646 has a GC-to-AT transition, converting an arginine to an ochre stop codon. The search of the GenBank, PIR and SWISS-PROT databases was done at the National Center for Biotechnology Information using the BLAST network service.

Fraction of identities with UNC-30

a	1	10	20	30	40	50															
	CTTCGAAGAGTCTCGTCTAGGATCAAAATCTTTGCGCTTCAATTAATCCGATCACTGA						57														
	CTTTGCGTAAGACGGTAATAATCTTGGGGATTGAGGCGCCTCCGGCAAAAGATCAACAC						117														
	ATGGATGACAATACGGCCACACTGACTGCTATACACCAGCAGCAGCAGTCGATAACGATT						177														
	M	D	D	N	T	A	T	L	T	A	I	H	Q	Q	Q	Q	S	I	T	I	18
	CTCAAAACCTCTGGTATGTGTGGGCAATTTGGATCATCACTCTCTGCTCCCGACGACTC						237														
	L	K	P	S	G	M	C	W	P	I	G	S	S	L	S	A	P	R	R	L	38
	AATTTCATCTTCCCTCGCACCATTAAACCCACAATCCATATGCATTCAACTACTCGATTCC						297														
	N	F	I	F	P	R	T	I	N	P	Q	S	I	C	I	Q	L	L	D	S	58
	ACTACCCCTACGGATATACCACAAAACCTCCCAAGTTAGAGCTGCTGCTACGAGCTG						357														
	T	T	P	T	D	I	T	T	K	L	P	K	L	E	L	L	S	L	D	V	78
	AAACAAGAGCAGGATGACAATCATCTCGACACATCGAGCCCAACAGATTCAACTGGAAAT						417														
	K	Q	E	Q	D	D	N	H	L	D	T	S	S	P	T	D	S	T	G	N	98
	GGAAGTACGAATGGAGGAAAATACAGAAACCGAGAAGGAGGACACATTTTACGAGC						477														
	G	S	T	N	G	K	I	Q	K	P	R	R	Q	R	T	H	F	T	S	118	
	CATCAGCTAACCGAATTTGAAAACCTGGTCTCCCGAAATCGCTATCCGACATGGCTTGT						537														
	H	Q	L	T	E	L	E	N	W	F	S	R	N	R	Y	P	D	M	A	C	138
	CGAGAAGAAATCGCGCTCGGATCAGCTTGACCGAGCCCTCGAGTGAGAGTTTGGTTCAAG						597														
	R	E	E	I	A	V	W	I	S	L	T	E	P	R	V	R	V	W	F	K	158
	AATCGACGTGCCAAATGGAGGAACCGCAAGCAAACTATGTGATGCAACGGCGAGGGC						657														
	N	R	R	A	K	W	R	K	R	E	R	N	Y	V	I	D	N	G	Q	G	178
	ACCACGAAGGTACCGCGCAAAAGTTTGGATCCACTTGGCTCACTTCAGAACAGTTTCCA						717														
	T	T	K	V	T	A	Q	S	L	D	P	L	G	S	L	Q	N	T	F	P	198
	CAGACGTTGTTCAGAGCAGCAGCTAGCTAGACGACTCTGCAGTAACGTCATCTCTCT						777														
	C	T	L	L	Q	Q	S	S	S	S	Q	L	D	D	S	A	V	T	S	S	218
	TTCTATGGATACGGTGGCGCGTGGCAACAGATCCATATCTACCCAGAAACAAATCAAC						837														
	F	Y	G	Y	G	G	A	W	Q	Q	N	P	Y	Y	S	R	N	N	Q	T	238
	ACGTTCACCTGGCAGATTAAACACAGTTTCAACAGATTCCAATGCTCTCCACAGCGGG						897														
	T	F	N	W	Q	I	K	P	Q	F	Q	T	I	P	M	S	P	T	T	A	258
	ACTAGTCGATTTTCAGCTGCTGCAAAATTTGGCACTCTACCAACAGCCCAAGCTGCATTC						957														
	T	S	R	F	S	T	A	N	L	A	P	L	P	T	A	Q	A	A	F	278	
	TCCACATCTGCCAGCTCATCAACGACAAGCTCAAAATTAATGACGGTCTCTCGAATCC						1017														
	S	T	S	A	T	S	S	N	D	K	L	K	L	M	D	G	L	S	N	S	298
	CTTCTCTCATCGCTCGGCCAACCTTATCAACCTGTGCTAGTACAGTGACCACTTTAGTTT						1077														
	L	S	S	L	G	Q	P	Y	Q	P	Q	C	Y	S	G	P	L	T	*	318	
	TTTAGCCACTTGAAAACTTTTATTTTGTTCGTTCTCTCCACCCGATCTCACATCCCAA						1137														
	AGCTTTTACTTTTGTTCCTCGCCTTTCAACTTTACTACTGTTCATTAACAAATTA						1197														
	AAAAGTATGAAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA						1257														

b	UNC-30	STOP (e646) E (e165)
	PRQRTHFTSHQLTELENNFWRNRYPDMAECREIAVWISLTPRVRVWFANRRAKWKRE	
	H-1 H-2 H-3	
Otd	Q--E--T--RA--DV--AL-GKT---IFM---V-LK-N-P-S--Q-----C-QQ-	35/60
Pax-3	Q--S--T--AE--E--RA-E-TH---IYT---L-QRAK---A-Q---S---R---QA	35/60
Otx1	Q--E--T--RS--DV--AL-AKT---IFM---V-LK-N-P-S--Q-----C-QQQ	34/60
UNC-4	R--T--N-SGW--E--SA-EASH---VFM---AL-MRLD-L-S--Q---Q-----	34/60
Gsd	K--H--I--DE--EA---L-QETK---VGT---QL-RRVH-R-EK-E-----RQK	32/60
Prd	Q--C--T--SAS--D--RA-E-TQ---IYT---L-QRTN---A-IQ---S---RL--QH	32/60
Bcd	---T--T--S-IA---QH-LQG---LTAPRLADLSAKLA-GTAQ-KI-----RRHKIQ-	24/60
Antp	RK-G-QTY-RY-TL---KE-HFN--LTRRR-I---HALC---RQIKI--Q---M-WK-EN	24/60

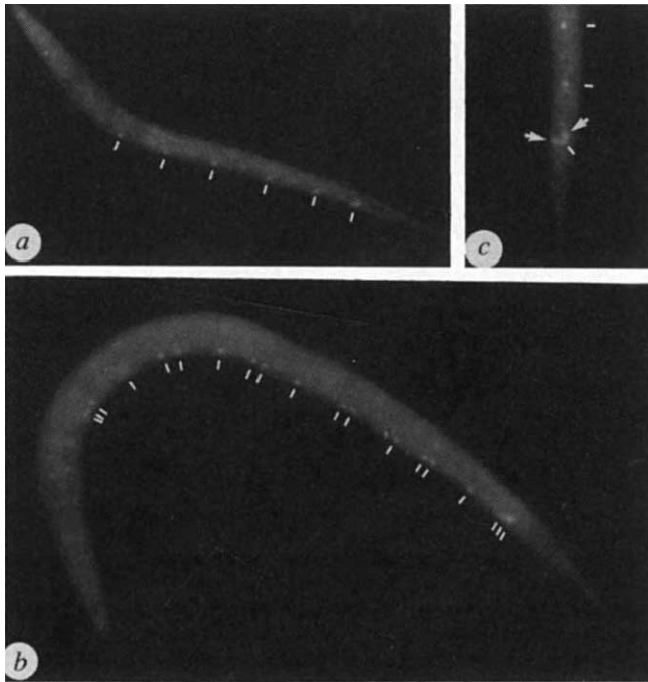


FIG. 3 Spatial distribution of the UNC-30 protein. *a*, L1 worm, lateral view. The six DD neurons are marked by the bars. *b*, L2 worm, lateral view. The six DD neurons and 13 VD neurons are marked by the bars. *c*, Preanal ganglion of an L1 worm, ventral view. The bars point to the DD neurons, and the arrowhead point to PVPs. We identified the cells that expressed the UNC-30 protein based on their positions and numbers. Both the D neurons and the PVPs were strongly stained. Four cells in the anterior region expressed the UNC-30 protein only weakly. These cells included a pair of cells located bilaterally in the lateral ganglia above the posterior bulb of the pharynx and identified as ASGR and ASGL³. The other two cells were located along the midline, between the anterior and posterior bulbs of the pharynx; the ventral midline cell appears to be RIH, and the dorsal midline cell is likely to be RID³.

METHODS. We expressed both the N-terminal and C-terminal portions of the UNC-30 protein in *E. coli* using the pET expression system (Novagen). To generate an N-terminal UNC-30 fusion protein, we first engineered an NcoI site at the putative ATG initiation codon. A XhoI site is present at nucleotide 585 of the cDNA. We then ligated in-frame the 465 bp 5' NcoI-XhoI 3' fragment of the cDNA into pET21d, which resulted in the production of the N-terminal 153 amino-acid residues. A construct for expressing the C-terminal 165 amino-acid residues of the UNC-30 protein was made by ligating in-frame the 565 bp 5' XhoI-HindIII 3' fragment of the cDNA into pET21b; the HindIII site is at nucleotide 1,143 and 60 bp downstream of the TAG. Both constructs resulted in overproduction of proteins of the predicted sizes following the experimental procedure provided by the manufacturer. Inclusion bodies containing overexpressed fusion proteins were isolated according to the instructions provided by Novagen. Proteins were fractionated by SDS-PAGE and UNC-30 fusion proteins were eluted by electrophoresis. Rabbits were injected with about 0.5 mg of antigen and boosted every 4 weeks. After three rounds of boosting, antisera were obtained and tested by immunostaining. Worms were fixed in 2% paraformaldehyde for 1 h and permeabilized according to the procedure of ref. 30. The UNC-30 antisera were preabsorbed against acetone powders of *E. coli* and of *unc-30(e2327)* worms, which are deleted for part of the *unc-30* coding region (see Fig. 1), and used at 1:200 dilution. Fixed worms were incubated with primary antibodies at 4 °C overnight, washed for 2–3 h and then incubated with 5% fluorescein isothiocyanate-conjugated (FITC) goat-anti-rabbit antibodies (Cappel). Animals were mounted in DABCO (1,4-diazabicyclo[2.2.2]octane) and viewed with a Zeiss Axiophot microscope using a standard Zeiss FITC filter set.

because we could not identify any potential consensus splice site in the upstream genomic sequence, we believe that this 1.3 kb cDNA represents a full-length *unc-30* transcript.

To identify the sites of *unc-30* action, we raised antibodies against UNC-30 protein expressed in *Escherichia coli* (see Fig. 3

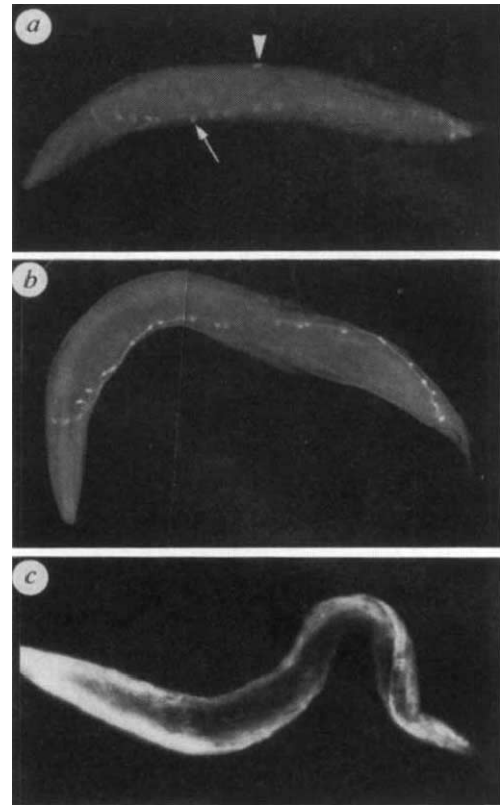


FIG. 4 Ectopic expression of GABA induced by ectopic expression of *unc-30*. *a*, A young adult worm carrying a *Pmec-7-unc-30* transgene. The mechanosensory neurons including ALML (arrowhead) as well as the normal GABAergic cells (one of which is indicated by the arrow) were stained with an anti-GABA antiserum. *b*, A control adult worm carrying a transgene with the *hsp16-2* and *hsp16-41* promoters but not the *unc-30* gene and subjected to heat-shock. An anti-GABA antiserum stained only the normal set of GABAergic neurons. *c*, An adult worm carrying a transgene of both *Phsp16-2-unc-30* and *Phsp16-41-unc-30* and subjected to heat-shock. This animal showed extensive staining with an anti-GABA antiserum, most strikingly in neurons and their processes. Occasionally, in other such animals we also observed GABA induction in non-neuronal cells, such as somatic gonad cells and pharyngeal muscle cells. Animals carrying this transgene but not subjected to heat-shock looked like the animal in *b*.

METHODS. The full-length *unc-30* c16 cDNA was inserted behind the promoters of *mec-7* in plasmid pPD52.102, of *hsp16-2* in pPD49.78, and of *hsp16-41* in pPD49.83 (gifts from A. Fire) to produce *Pmec-7-unc-30*, *Phsp16-2-unc-30* and *Phsp16-41-unc-30*, respectively. The *Pmec-7-unc-30* construct was coinjected into N2 animals with the dominant roller marker pRF4⁸ at a concentration of 50 µg ml⁻¹ of each, and roller lines were established. Roller worms of mixed stages were collected and fixed in 4% paraformaldehyde and 1% glutaraldehyde and stained with anti-GABA antiserum as described²⁰. The two heat-shock inducible promoters showed different and to a large extent complementary patterns of expression¹⁶. For this reason, the *Phsp16-2-unc-30* and *Phsp16-41-unc-30* were injected together into N2 animals with the dominant roller marker pRF4⁸ at the concentration of 30 µg ml⁻¹ of each, and roller lines were established. Heat-shock was at 33 °C for 1 h. Heat-shocked animals were allowed to recover for at least 3 h at 20 °C. The worms were then collected, fixed and stained with an anti-GABA antiserum.

legend). Immunocytochemical studies revealed that the UNC-30 protein is found in the nuclei of the D neurons (Fig. 3). The 19 D neurons consist of 6 dorsal-D (DD) neurons, which are generated embryonically, and 13 ventral-D (VD) neurons, which are added during the first larval (L1) stage³. In L1 worms anti-UNC-30 antisera stained the 6 DD neurons (Fig. 3*a*), and in L2 worms the antisera stained the 6 DD neurons and the 13 VD neurons (Fig. 3*b*). UNC-30 protein was maintained at a rela-

tively low level in the D neurons in L3 and later stage animals (data not shown). Anti-UNC-30 antisera also stained a pair of nuclei in the preanal ganglion in both L1 and L2 animals. The positions of these nuclei corresponded to those of the PVP neurons (Fig. 3c), suggesting that *unc-30* might be necessary for PVP development or function, which is consistent with the finding that in *unc-30* mutants a PVP cell-surface antigen is absent (H. Bhatt and E. Hedgecock, personal communication). The function, lineage and neurotransmitter of the PVPs are not obviously related to those of the D neurons³. In addition, four nuclei in the head of L1 and L2 animals were weakly stained by the anti-UNC-30 antisera; these cells were identified as the sensory neurons ASGL and ASGR and the interneurons RIH and RID (see Fig. 3 legend).

As noted above, *unc-30* is essential for the expression of certain terminally differentiated characteristics of the type D neurons. Is *unc-30* function sufficient to induce such characteristics in other cell types? To answer this question, we induced the ectopic expression of *unc-30* using the promoter of the *mec-7* gene, which is expressed in the six mechanosensory neurons¹⁵. These mechanosensory neurons normally do not express GABA. In transgenic animals carrying the *Pmec-7-unc-30* transgene the mechanosensory neurons stained with GABA antisera (Fig. 4a, b), and these transgenic animals were insensitive to gentle touch, indicating that mechanosensory neuron function was lost. Ectopic expression of *unc-30* also could induce ectopic GABA expression in many other types of cells. Using the *hsp16-2* and *hsp16-41* promoters, which together cause heat-inducible expression in nearly all somatic tissues¹⁶, we made transgenic animals carrying both *Phsp16-2-unc-30* and *Phsp16-41-unc-30*, two DNA constructs in which *unc-30* cDNA is driven by *hsp16-2* or *hsp16-41*, respectively (see Fig. 4 legend). Such animals produced UNC-30 protein in most neurons and in most non-neuronal cells following heat-shock (data not shown). In these animals many neurons contained GABA (Fig. 4c), and in occasional animals non-neuronal tissues also contained GABA. Although we do not know whether other differentiated features specific to the type D neurons were also induced, these experiments indicate that in certain cell types *unc-30* can induce the production of the D neuron neurotransmitter, GABA.

That *unc-30* is not expressed in the seven non-D type GABAergic neurons is consistent with the finding that these other GABAergic neurons appear to be unaffected in *unc-30* mutants². Thus, *unc-30* is not necessary for GABAergic neuronal differentiation itself but rather is required specifically for the development of the type D neurons. That UNC-30 is also present in six non-GABAergic non-D type neurons suggests that *unc-30* may control the differentiation of those neuron types. If so, *unc-30* must act in combination with different genes in different neuron types to induce their distinctive differentiated features. For example, the type-D neurons may express genes that act with *unc-30* to cause GABA expression in these cells, whereas the six non-GABAergic cells that contain UNC-30 protein, such as the PVPs, may express other genes that act with *unc-30* to cause the expression of other neuronal traits. In contrast, the widespread expression of GABA in response to ectopic *unc-30* expression suggests that UNC-30 protein may be sufficient to induce GABA expression in most cell types, in which case the six non-GABAergic UNC-30-positive neurons may express one or more negative differentiation factors that prevent GABA expression by inhibiting UNC-30 function. □

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dpp induces mesodermal gene expression in *Drosophila*

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INDUCTIVE interactions between germ layers are an essential feature of the development of many organisms. In several species these interactions are mediated by members of the transforming growth factor- β (TGF β) family^{1,2}. In amphibians, different concentrations of activin can induce different types of mesoderm in the animal cap assay^{3–5}. In *Drosophila*, a member of the TGF β family, *decapentaplegic* (*dpp*)^{6,7}, acts as an inductive signal. Midway through embryogenesis, *dpp* is expressed in the visceral mesoderm, and enhances the expression of the homeotic gene *labial* in the underlying midgut endoderm^{8,9}. Earlier in development, however, *dpp* expression is limited to the dorsal ectoderm¹⁰. At this stage in development, *thickveins*, a *dpp* receptor^{11–13}, is expressed in the mesoderm^{12,13}, and this suggests that ectodermal *dpp* might not only be required for development of dorsal ectoderm^{14,15}, but could also act inductively to mediate pattern formation in the underlying mesoderm. Here we show, by expressing *dpp* ectopically in the ectoderm and mesoderm and by examining *dpp* null mutant embryos, that *dpp* regulates expression of mesodermal genes.

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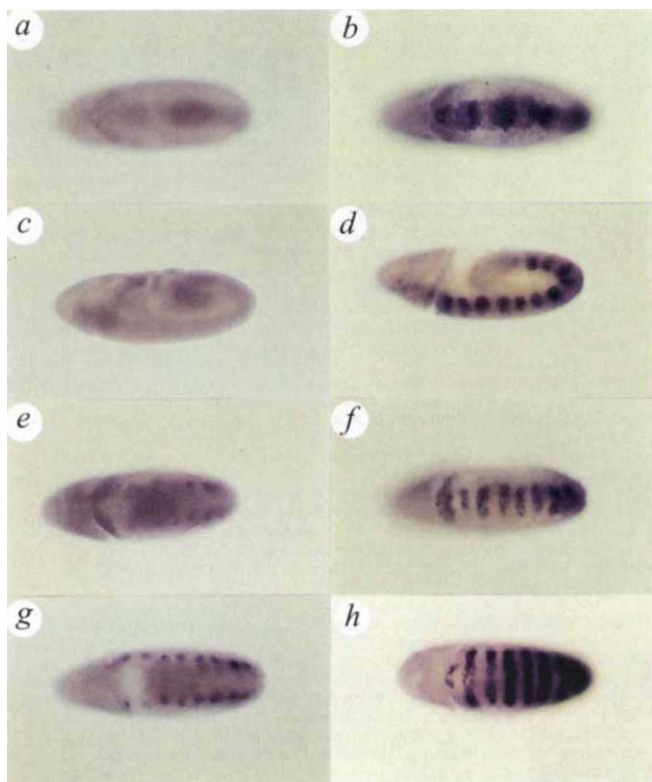


FIG. 1 Expression of *bap* mRNA in wild-type embryos (a, c, e, g) and in embryos carrying the *twist-GAL4;UAS-dpp* constructs (b, d, f, h). In the wild type, *bap* is first detected as faint patches of dorsal expression in early stage 10 (e), which are intensely stained by late stage 10 (g). There is no expression in the invaginating ventral furrow (a), or as the germ band extends (c). In embryos carrying *twist-GAL4;UAS-dpp*, expression begins in cells of the invaginating ventral furrow (b), with enhanced staining in a series of patches along the longitudinal axis. As the germ band extends (d), this pattern evolves into a sequence of stripes, which extend as the mesoderm migrates dorsally (f), to include the normal domain of *bap* expression as well as the ventral domain of ectopic *bap* expression (h). Note that *dpp* RNA in the visceral mesoderm of wild-type embryos is first detected several hours after gastrulation at mid stage 11 and is not relevant to the onset of *bap* expression. METHODS. Embryos carrying the GAL4;UAS constructs were generated by mating two homozygous stocks: *yw; twist-GAL4* (M.K.B. and M.B., manuscript in preparation) $\times$ *w; UAS-dpp*²¹. All embryos generated from this cross carry one copy of both constructs. *In situ* hybridizations were done as previously described²⁷ using a 1.5 kb *bap* cDNA probe (kindly provided by M. Frasch) on wild-type (*Oregon R*) and *twist-GAL4;UAS-dpp* embryos.

The early expression of *dpp* is restricted to a dorsal domain of the ectoderm where it is required for the development of the dorsalmost parts of the epidermis^{14–16}. In the mesoderm, the expression of *tinman* (*tin*)^{17–19} and *bagpipe* (*bap*)¹⁹, two genes required for the formation of visceral mesoderm, is confined to a dorsal domain that underlies the early ectodermal expression of *dpp* (Fig. 1e). In the case of *tin*, however, dorsal restriction is preceded by expression throughout the invaginating mesoderm^{17,19}. Although expression of *bap* depends on *tin*¹⁹, in normal development *bap* is never expressed ventrally in the mesoderm, that is, in the domain of early *tin* expression (Fig. 1a, c). To investigate whether the dorsally restricted expression of *bap* within the *tin* domain depends on an additional requirement for *dpp*, we used the GAL4 targeted expression system²⁰ to promote *dpp* expression throughout the early mesoderm using *twist-GAL4* (M.K.B. and M.B., manuscript in preparation) and *UAS-dpp*²¹. In embryos carrying these constructs, *bap* expression is initiated prematurely as mesodermal cells invaginate through the ventral furrow (Fig. 1b). Thus, in experimental embryos, as

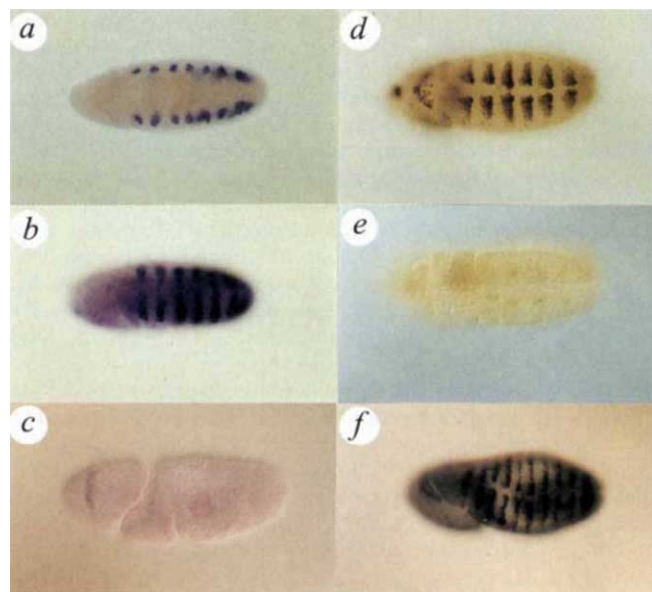


FIG. 2 Patterns of mesodermal gene expression in wild-type embryos (a, d), in embryos carrying *69BGAL4;UAS-dpp* (b, e) and in *dpp* null mutant embryos (c, f). At stage 11, in wild-type embryos, *bap* mRNA is expressed in a series of prominent mesodermal patches underlying the tracheal pits (a). Where *dpp* is ectopically expressed throughout the ectoderm (b), this expression is extended ventrally to form a series of stripes (see Fig. 1h). In *dpp* null mutant embryos, *bap* expression is lost, except for weak staining in the head and hindgut (c). *pox meso* protein is expressed in a series of ventral mesodermal stripes from stage 10 in wild-type embryos (d, stage 11). In embryos where *dpp* is expressed in ventral as well as dorsal ectoderm, mesodermal *pox meso* expression is abolished (e). A similar phenotype is seen in embryos carrying *twist-GAL4;UAS-dpp*. Conversely, in *dpp* null mutant embryos (f), mesodermal *pox meso* expression extends over the entire embryonic circumference.

METHODS. *69BGAL4;UAS-dpp* embryos were generated from the two stocks *w;69BGAL4* $\times$ *w;UAS-dpp*. All embryos resulting from this cross carried one copy of both constructs. *dpp* null mutant embryos were generated from a *dpp*⁶¹ stock. Homozygous *dpp*⁶¹ embryos were identified by their abnormal morphology and, in particular, the deep cephalic furrow. *In situ* hybridization as in Fig. 1. Antibody staining as previously described²⁸, using a 1/100 dilution of anti-*pox meso* antibody kindly provided by M. Noll.

cells that express *tin* encounter *dpp* expressed by themselves and their neighbours, they turn on the expression of *bap*. In normal development, mesodermal cells first encounter cells secreting the *dpp* product (Dpp) as they migrate under dorsal ectoderm at stage 9, and soon these dorsal mesodermal cells begin to express *bap* in wild-type embryos¹⁹ (Fig. 1e, g). To test whether ectodermally derived Dpp can elicit expression of *bap* in the mesoderm, we used a second, ectodermally expressed GAL4 construct, *69B*^{20,22}, to promote *dpp* expression throughout the ectoderm from late stage 9 onwards. In these embryos, *bap* expression is extended ventrally (Fig. 2a, b). We do not see premature expression of *bap* in the ventral furrow because gastrulation occurs before *dpp* is expressed under the control of *69B*. From these observations we conclude that cells of the *tin* domain have an additional requirement for Dpp to elicit the expression of *bap* and that ectodermally derived Dpp can provoke *bap* expression in underlying mesodermal cells.

If *dpp* is required for activation of dorsally restricted mesodermal genes, we reasoned that it might also be required for repression, dorsally, of genes whose expression is normally confined to ventral mesoderm. To test this idea, we looked at the expression of *pox meso*, a putative transcription factor that is expressed in segmentally repeated stripes in the mesoderm immediately underlying the neurogenic ectoderm and not extending

dorsally beyond the ventral margin of the tracheal pits²³ (Fig. 2d). Thus mesodermal *pox meso* in normal embryos is confined to a region underlying ectoderm where *dpp* is not expressed in early embryogenesis. In embryos carrying either the *twist-GAL4;UAS-dpp* (data not shown) or *69BGAL4;UAS-dpp* constructs, mesodermal expression of *pox meso* is suppressed (Fig. 2e). Thus ectopic Dpp in either ventral mesoderm, or adjacent ectoderm, is sufficient to abolish *pox meso* expression in the mesoderm. In wild-type embryos, such an inhibitory effect would be sufficient to restrict *pox meso* to its normal domain of ventral mesodermal expression.

A clear prediction from the results of ectopic expression of *dpp* is that removal of *dpp* should produce opposite effects. In embryos lacking *dpp*, there should be no expression of *bap* and, conversely, expression of *pox meso* should be enhanced. We therefore examined expression of *bap* and *pox meso* in *dpp* null mutant embryos. Expression of *bap* in the mesoderm is abolished and staining can only be detected in the head and hindgut regions (Fig. 2e). By contrast, mesodermal expression of *pox meso* protein expands around the circumference of *dpp* null embryos (Fig. 2f). These results, together with the ectopic expression studies, indicate that *dpp* induces *bap* expression and suppresses *pox meso* expression in the mesoderm. In normal development this is sufficient to restrict the expression of *bap* to dorsal and *pox meso* to ventral mesoderm.

A conspicuous consequence of ectopic *dpp* expression in either ectoderm or mesoderm is that epidermis in such embryos is dorsalized. Although this is not unexpected for embryos carrying *69BGAL4;UAS-dpp*, which provides a source of Dpp throughout the ectoderm^{20,22}, it is striking that it also occurs when *dpp* is expressed in the mesoderm. *twist-GAL4* promotes expression in mesectoderm as well as mesoderm (M.K.B. and M.B., manuscript in preparation). To avoid the complication of mesectodermally expressed *dpp* affecting ventral ectoderm, we expressed *dpp* under the control of *24BGAL4*, which promotes expression from stage 10 onwards, in the mesoderm^{20,24}. Cuticles of embryos carrying *24BGAL4;UAS-dpp* are virtually wild type (Fig. 3a). However, when the dose of mesodermally expressed *dpp* is increased by doubling the number of copies of *UAS-dpp*, the cuticle is clearly dorsalized (Fig. 3b, c). We conclude that, under appropriate conditions, *dpp* can operate across germ layers in either direction: both from the mesoderm (in experimental embryos) to dorsalize the ectoderm and from the ectoderm (in normal embryos) to regulate patterns of mesodermal gene expression.

Transplantation experiments have shown that fates of mesodermal cells taken from the invaginating ventral furrow are undecided²⁵. Additional information is required to subdivide the mesoderm into progenitors of different mesodermal derivatives, and this information is closely linked to position²⁵. Experiments by Bock in embryos of another insect²⁶, suggested that one source of this information might be the ectoderm over which invaginated mesoderm migrates, and to which, during early embryogenesis, it tightly adheres. Here we have shown that an ectodermally derived signalling molecule, Dpp, acts across germ layers to regulate gene expression in the mesoderm. Dpp activates a dorsally expressed gene, *bap*, and represses a ventrally expressed gene, *pox meso*. The ectodermal distribution of *dpp* in normal embryos is sufficient to ensure that *bap*, which is required for the formation of visceral musculature¹⁸, is expressed only in a dorsal mesodermal domain, whereas *pox meso* is confined to ventral mesoderm underlying the neurogenic ectoderm. The fact that mesodermal expression of *dpp* can elicit *bap* expression in the ventral furrow before mesodermal cells migrate beneath the ectoderm reinforces our conclusion that, in wild-type embryos, ectodermal Dpp may act directly to regulate *bap* expression, rather than through some other signalling pathway. Other experiments indicate that ectodermal expression of *wingless* can influence mesodermal gene expression (M.K.B. *et al.*, manuscript in preparation). Regulative interactions of this kind

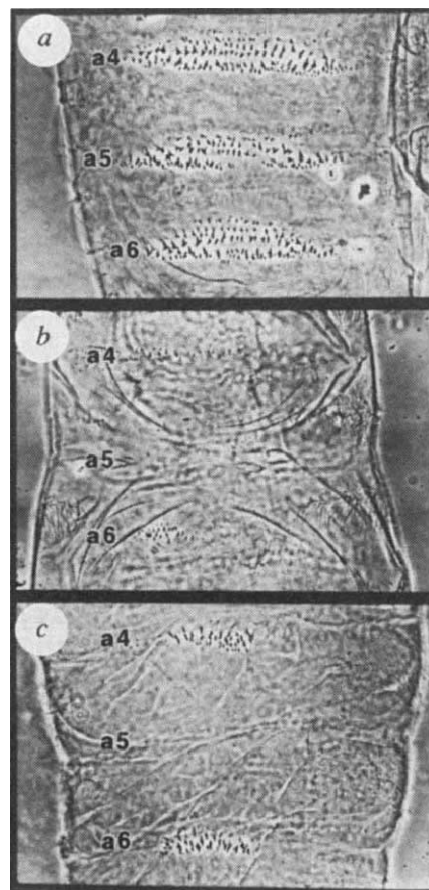


FIG. 3 Cuticles of animals that express ectopic *dpp* in the ectoderm and mesoderm. The ventral surfaces of abdominal segments 4, 5 and 6 (a4, a5, a6) are shown. a, Denticle bands in animals that express one copy of *UAS-dpp* in the *24BGAL4* mesodermal pattern raised at 29 °C are not significantly different from denticle bands in wild-type cuticles. b, Denticle bands in an embryo expressing two copies of *UAS-dpp* in the *24BGAL4* pattern at 29 °C. Notice how denticle bands a4 and a6 are greatly reduced and band a5 is absent. c, Denticle bands in an embryo expressing one copy of *UAS-dpp* in the *69BGAL4* ectodermal pattern at 25 °C. Notice how the phenotype is similar to b even though a quantitatively weaker *UAS-dpp* line was used in c and the embryos were raised at a lower temperature. The specific line of *UAS-dpp* used here provides less expression of *dpp* than the lines used in b as determined by the degree of lethality and the severity of phenotypes caused by these lines in several different *GAL4* expression patterns (K.S.-H. and F.M.H., unpublished observations). The *GAL4;UAS* system is intrinsically temperature sensitive; at higher temperatures (such as 27 °C or 29 °C) more severe phenotypes are observed and higher levels of β -galactosidase are generated from a *UAS-lacZ* target gene²⁰, than at lower temperatures (such as 18 °C or 22 °C) (K.S.-H. and F.M.H., unpublished observations).

METHODS. Cuticles were prepared as described previously²⁹.

divide the mesodermal primordium into spatially organized subsets of cells, which are the progenitors of different mesodermal derivatives. In this instance a *dpp*-dependent mechanism allocates cells to form visceral mesoderm.

Clearly, however, the pattern of *bap* expression shows that its activation does not depend only on the presence of ectodermal *dpp*. Although in wild-type embryos *dpp* is expressed throughout the dorsalmost ectoderm, *bap* is expressed in a discontinuous pattern of dorsal patches underlying the developing tracheal pits. Thus there must be additional factors, perhaps intrinsic to the mesoderm, which influence the competence of mesodermal cells to respond to signals such as *dpp*. These domains of competence are also revealed in experimental embryos, where pan meso-

dermal expression of *dpp* provokes *bap* expression in the ventral furrow in patches (Fig. 1b) that evolve into stripes aligned with the future tracheal pits (Fig. 1d, f, h).

Our experiments provide evidence for a molecular mechanism by which the inductive process proposed in ref. 26 might function in patterning the mesoderm. Ectodermally derived signals such as *dpp* act with machinery that limits the competence of mesodermal cells to respond. This interaction leads to spatially organized patterns of mesodermal gene expression which initiate the segregation of different mesodermal derivatives in proper register with the overlying ectoderm. □

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Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase

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THE cytoplasmic focal adhesion protein-tyrosine kinase (FAK) localizes with surface integrin receptors at sites where cells attach to the extracellular matrix. Increased FAK tyrosine phosphorylation occurs upon integrin engagement with fibronectin. Here we show that adhesion of murine NIH3T3 fibroblasts to fibronectin promotes SH2-domain-mediated association of the GRB2 adaptor protein and the c-Src protein-tyrosine kinase (PTK) with FAK *in vivo*, and also results in activation of mitogen-activated protein kinase (MAPK). In v-Src-transformed NIH3T3, the association of v-Src, GRB2 and Sos with FAK is independent of cell adhesion to fibronectin. The GRB2 SH2 domain binds directly to tyrosine-phosphorylated FAK. Mutation of tyrosine residue 925 of FAK (YENV motif) to phenylalanine blocks GRB2 SH2-domain binding to FAK *in vitro*. Our results show that fibronectin binding to integrins on NIH3T3 fibroblasts promotes c-Src and FAK association and formation of an integrin-activated signalling complex. Phosphorylation of FAK at Tyr 925 upon fibronectin stimulation creates an SH2-binding site for GRB2 which may link integrin engagement to the activation of the Ras/MAPK signal transduction pathway.

To study the role of FAK in integrin signalling, we affinity-purified anti-FAK antibodies, which immunoprecipitated a 116K protein (M_r 116,000) from quiescent NIH3T3 cells which comigrated with FAK immunoprecipitated by anti-FAK monoclonal antibody 2A7 (Fig. 1a, lanes 3 and 4)¹. Anti-phosphotyrosine (anti-P.Tyr) blotting of quiescent NIH3T3 lysates showed

that FAK was tyrosine-phosphorylated (Fig. 1a, lanes 5–7)^{2–4}. Oncogenically active Src is complexed with FAK in chicken embryo fibroblasts, through binding of its SH2 domain to the Tyr 397 FAK autophosphorylation site^{5,6}. The c-Src SH2 domain bound tyrosine-phosphorylated FAK in plastic-grown quiescent NIH3T3 lysates (Fig. 1b). When NIH3T3 cells were detached by limited trypsin treatment before lysis, FAK was rapidly dephosphorylated and c-Src SH2 binding was dramatically reduced (Fig. 1b).

Although c-Src SH2-domain binding to FAK was readily detected *in vitro*, no association was detected between c-Src and FAK *in vivo* (Fig. 1c, lane 2). Overexpression of chicken c-Src in NIH3T3 cells with or without vanadate treatment resulted only in weak c-Src-FAK association (Fig. 1c, lanes 7 and 8). The weak association may be due to negative regulation of c-Src in quiescent cells by Csk⁷ or to differential compartmentalization of c-Src and FAK⁸.

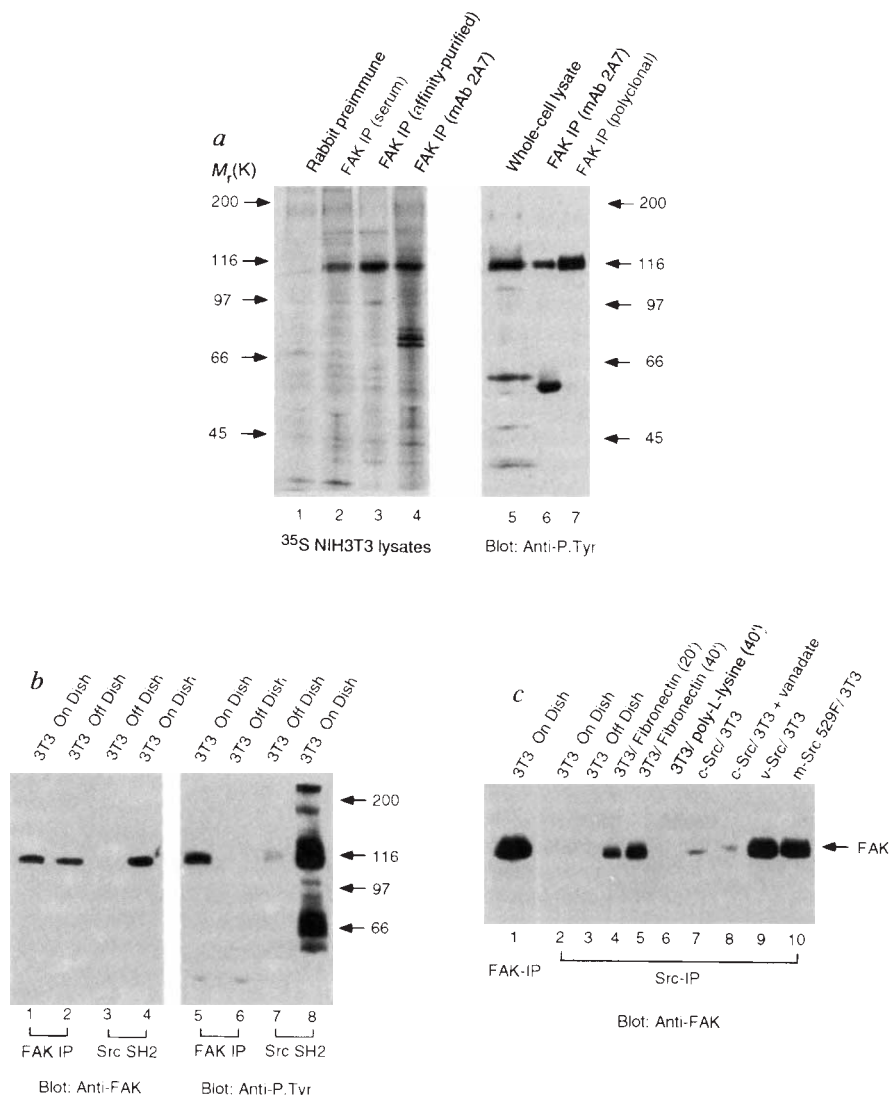
In contrast, *in vivo* association of FAK with c-Src was detected when quiescent NIH3T3 cells were replated onto fibronectin-coated (Fig. 1c, lanes 4 and 5) but not poly-L-lysine-coated dishes (Fig. 1c, lane 6). Cells plated onto fibronectin began to adhere and spread within 20 min, whereas on poly-L-lysine cells attached but remained 'rounded'. In v-Src or F529Src-transformed NIH3T3 cells, FAK was constitutively associated with Src (Fig. 1c, lanes 9 and 10); this association was independent of cell adhesion (data not shown). This constitutive FAK/v-Src association may bypass the normal integrin-activated cellular signals that promote FAK/c-Src interactions.

Fibronectin stimulation increases FAK tyrosine phosphorylation^{2–4,9,10} and FAK PTK activity⁴. Our results suggest that c-Src and FAK form an integrin-activated signalling complex. To investigate whether other signalling proteins associate with FAK following integrin activation, FAK was immunoprecipitated from lysates of NIH3T3 cells grown on plastic, detached from plastic, detached and replated onto fibronectin or poly-L-lysine, and from attached or detached v-Src 3T3 cells. The immunoprecipitates (IPs) were analysed first by anti-FAK (Fig. 2a) and then by anti-P.Tyr blotting (Fig. 2b). Nearly equal amounts of FAK were immunoprecipitated from the various 3T3 lysates (Fig. 2a). Upon reprobing this blot with anti-P.Tyr antibodies, several P.Tyr-containing proteins (55K–110K) were detected in FAK IPs from NIH3T3 cells replated on fibronectin (Fig. 2b, lanes 4 and 5) and in v-Src 3T3 cells (Fig. 2b, lanes 8 and 9), but not in IPs from quiescent NIH3T3 cells (Fig. 2b, lane 2) or cells replated on poly-L-lysine (Fig. 2b, lanes 6 and

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FIG. 1 FAK association with c-Src *in vitro* and *in vivo*. **a**, IPs with pre-immune serum (lane 1), anti-FAK antiserum (lane 2), affinity-purified anti-FAK antibodies (lanes 3 and 7) or with anti-FAK mAb 2A7 (lanes 4 and 6) were made from ^{35}S -methionine-labelled NIH3T3 lysates (lanes 1–4) or from unlabelled quiescent NIH3T3 lysates (lanes 6 and 7). NIH3T3 whole cell lysate was also analysed (lane 5). IPs were visualized by autoradiography (lanes 1–4) or analysed by anti-P.Tyr immunoblotting (lanes 5–7). The ~55K band in lane 6 is due to mouse IgG crossreactivity with the secondary antibody ECL detection reagents. **b**, IPs with either anti-FAK antibodies (lanes 1, 2, 5 and 6) or *in vitro* binding reactions with GST–Src SH2-domain fusion protein (lanes 3, 4, 7 and 8) were prepared from lysates of NIH3T3 cells grown on plastic (lanes 1, 4, 5 and 8) or detached from plastic (lanes 2, 3, 6 and 7) and analysed by anti-FAK (lanes 1–4) followed by anti-P.Tyr immunoblotting (lanes 5–8). **c**, IPs with either anti-FAK antibodies (lane 1) or anti-Src mAb 2-17 (lanes 2–10) were prepared from NIH3T3 cells grown on plastic (lanes 1, 2, 7–10), detached from plastic (lane 3), detached from plastic and replated onto fibronectin-coated dishes (lanes 4 and 5) or poly-L-lysine-coated dishes (lane 6). NIH3T3 cells overexpressing chicken c-Src (lane 7) were incubated with Na_3VO_4 before lysis (lane 8). NIH3T3 cells transformed by v-Src (lane 9) or by activated (mutation at Y529 to F) mouse c-Src (lane 10) were used as positive controls for FAK coprecipitation with Src.

METHODS. The FAK C-terminal domain (Gly 853 end) was amplified by PCR using the mouse FAK cDNA as a template³ and cloned into pGEXKT for protein expression. The FAK residues were cleaved from GST with thrombin and used as antigen. Rabbit antibodies were purified over a crosslinked glutathione-agarose GST–FAK C-terminus affinity column. IPs were made using 1 mg total NIH3T3 lysate and ~2 μg IgG was used per IP and collected with protein A- or protein G-Sepharose. ^{35}S -methionine-labelled NIH3T3 cell lysates were prepared as described¹⁵. For replating experiments, the NIH3T3 cells were serum-starved overnight in DMEM containing 0.5% CS and replated onto fibronectin- ($10 \mu\text{g ml}^{-1}$) or poly-L-lysine ($200 \mu\text{g ml}^{-1}$)-coated dishes as described³. Cells were incubated at 37°C and, at either 20 or 40 min following plating, washed with cold PBS and lysed in 750 μl modified RIPA buffer. Total protein concentrations in clarified lysates were equalized before immunoprecipitation or *in vitro* binding assay. For either IPs or *in vitro* binding assays using 10 μg fusion



protein, incubations were for 2 h at 4°C followed by washing twice in 1 ml lysis buffer (lacking deoxycholate and SDS) and twice in 1 ml HNTG buffer¹². Samples were boiled, resolved on SDS/10% PAGE gels, transferred to Immobilon-PVDF membranes, immuno-blotted¹⁵ and visualized by enhanced chemiluminescent detection (ECL) (Amersham). For anti-P.Tyr immunoblotting, mAb 4G10 was used at $1 \mu\text{g ml}^{-1}$ and a polyclonal antiserum against the FAK C terminus³ was used for anti-FAK immunoblotting.

7). The association of other P.Tyr-containing proteins with FAK appeared to depend on increased FAK tyrosine phosphorylation, which occurs after integrin stimulation.

To determine whether the integrin-activated FAK signalling complexes contained other known SH2-containing proteins, we tested whether the SH2/SH3 adaptor protein GRB2 was present in FAK IPs by anti-GRB2 blotting. Only in fibronectin-replated NIH3T3 (Fig. 2c, lanes 4 and 5) and v-Src 3T3 cells (Fig. 2c, lanes 8 and 9) was GRB2 present in FAK IPs. To characterize the *in vivo* association of GRB2/FAK, GRB2 was immunoprecipitated directly from similarly prepared lysates (Fig. 2d). A prominent P.Tyr-containing 116K protein was present in GRB2 IPs from fibronectin-replated (Fig. 2e, lanes 12 and 13) and v-Src 3T3 cells (Fig. 2e, lanes 16 and 17), which was confirmed to be FAK by immunoblotting (Fig. 2f).

To test whether GRB2/FAK association in fibronectin-replated NIH3T3 cells required the GRB2 SH2 domain, and whether other SH2-containing proteins bound FAK, we assayed

in vitro binding with different glutathione-S-transferase (GST)–SH2 fusion proteins. Only the Src SH2 bound to FAK strongly in plastic-grown NIH3T3 lysates (Fig. 3a). Cell detachment before lysis caused FAK dephosphorylation and abolished Src SH2 binding (Fig. 3b). Replating onto fibronectin dramatically enhanced binding of the Src, GRB2, NCK phospholipase $\text{C}\gamma$, p85 and CSK SH2 domains to FAK (Fig. 3c). The binding of p85 SH2 to FAK agrees with the demonstration that FAK IPs from NIH3T3 cells contain phosphatidylinositol-3-OH kinase activity¹¹.

The enhanced binding of these SH2 domains to FAK *in vitro* depended on integrin engagement of NIH3T3 cells, because it was detected after replating onto fibronectin (Fig. 3c) but not onto poly-L-lysine (Fig. 3d). SH2 binding to FAK was also enhanced in v-Src 3T3 lysates (Fig. 3e), where binding was independent of integrin engagement and cell attachment (Fig. 3f).

FIG. 2 *In vivo* associations of GRB2 and P.Tyr-containing proteins with endogenous FAK are mediated by NIH3T3 cell adhesion to fibronectin. IPs were made with anti-FAK preimmune control (lane 1), affinity-purified anti-FAK antibodies (lanes 2–9), affinity-purified anti-GRB2 antibodies (lanes 10–17), and anti-GRB2 in the presence of excess peptide 'block' used to raise the GRB2 antiserum (lane 18). Lysates were prepared from serum-starved NIH3T3 cells grown on plastic (lanes 1, 2 and 10), detached from plastic and replated onto fibronectin-coated dishes for 20 min (lanes 3 and 11), detached from plastic and replated onto poly-L-lysine-coated dishes for 20 min (lanes 4 and 12) or 40 min (lanes 5 and 13), and from cells detached from plastic and replated onto poly-L-lysine-coated dishes for 20 min (lanes 6 and 14) or 40 min (lanes 7 and 15). Lysates were prepared from attached (lanes 8, 16 and 18) or detached (lanes 9 and 17) v-Src 3T3 cells. *a* and *f*, Anti-FAK blot of the IPs; *b* and *e*, anti-P.Tyr blots of the membranes shown in *a* and *f*. *c* and *d*, Anti-GRB2 immunoblots of the IPs shown in *a* and *f*.

METHODS. All IPs were performed with antibodies covalently attached to protein A-Sepharose. Bound antibodies were coupled with 20 mM dimethylpimelimidate in 0.2 M sodium borate, pH 9.0 and washed in 0.2 M ethanolamine, pH 8.0, followed by PBS. IPs were made as described in Fig. 1 legend. Membranes were cut and proteins from 45K to 200K were analysed in *a*, *b*, *e* and *f*. Proteins from 22K to 31K were analysed in *c* and *d* (GRB2 runs at ~27K). Membranes were stripped and reprobed with anti-P.Tyr antibody as for Fig. 1. GRB2 IPs were done with (C-23) anti-peptide GRB2 antibody against the C-terminal 23 amino acids of GRB2 (Santa Cruz Biotechnology) directly coupled to protein A-Sepharose. 50 µg of the GRB2 C-terminal peptide was used to block antibody binding. GRB2 immunoblots were performed with the GRB2 C-23 antibody at 400 ng ml⁻¹. Enhanced chemiluminescent exposure times: *a*, 2 s; *b*, 10 s; *c*, 5 min; *d*, 10 s; *e*, 2 min; and *f*, 2.5 min.

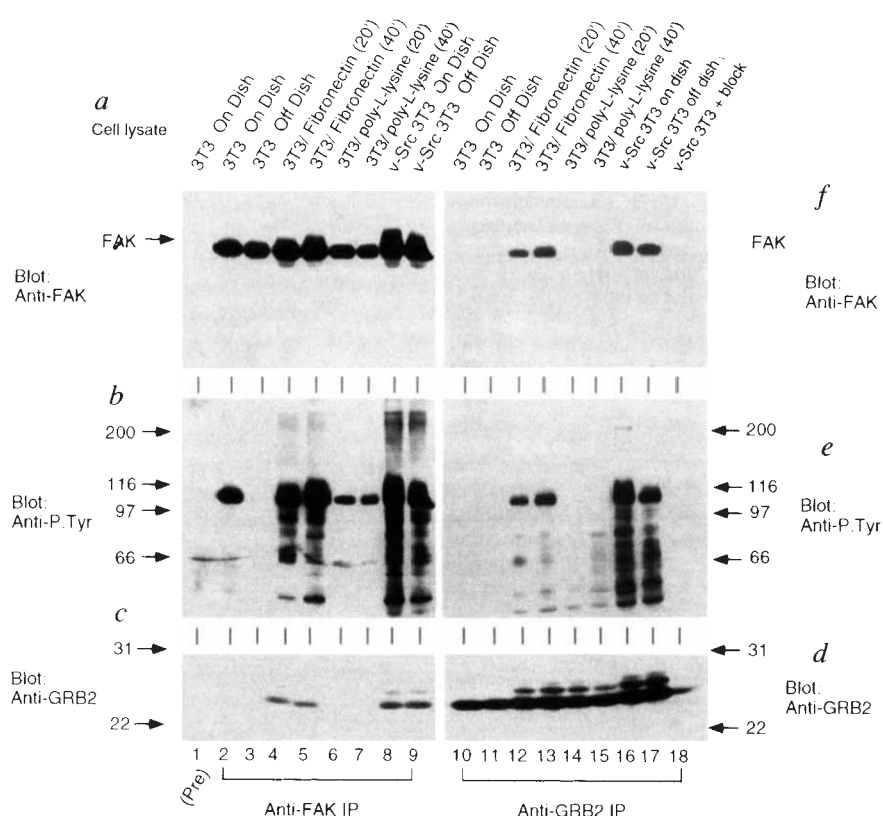
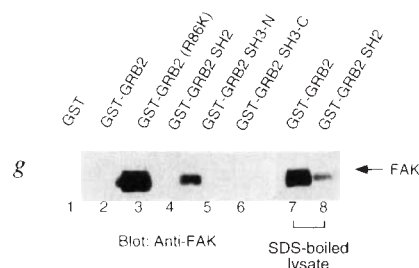
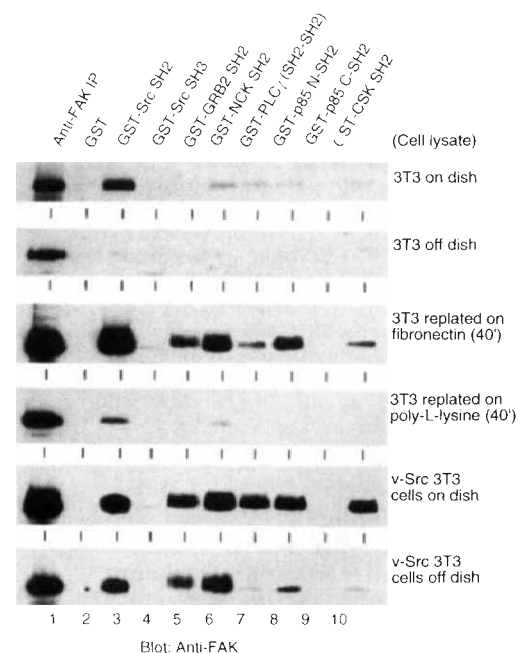


FIG. 3 The Src, GRB2, NCK, PLC γ , p85 and CSK SH2 domains associate with FAK after NIH3T3 cell plating on fibronectin; GRB2 binds to FAK directly. *a–g*, Anti-FAK IP control (lane 1) and *in vitro* binding assays with various GST fusion protein constructs: GST (lane 2), GST-Src SH2 (lane 3), GST-Src SH3 (lane 4), GST-GRB2 SH2 (lane 5), GST-NCK SH2 (lane 6), GST-PLC γ SH2-SH2 (lane 7), GST-p85 (N)SH2 (lane 8), GST-p85 (C)SH2 (lane 9) and GST-CSK SH2 (lane 10). *a*, Lysates from serum-starved NIH3T3 cells grown on plastic. *b*, Lysates from detached NIH3T3 cells. *c*, Lysates from NIH3T3 cells replated on fibronectin for 40 min. *d*, Lysates from NIH3T3 cells replated on poly-L-lysine for 40 min. *e*, Lysates from v-Src 3T3 cells grown on plastic. *f*, Lysates from detached v-Src 3T3 cells. *g*, Lysates from v-Src 3T3 cells in RIPA buffer (lanes 1–6) or from v-Src 3T3 SDS-boiled lysates (lanes 7 and 8). *In vitro* association with various GST fusion protein constructs: GST (lane 1), full-length GRB2 (lanes 2 and 7), full-length GRB2 R86K-SH2 mutant (lane 3), GRB2 SH2 (lanes 4 and 8), GRB2 SH3 (N) (lane 5), and GRB2 SH3 (C) (lane 6). In *a–g*, only the FAK region of the gel is shown.

METHODS. The GST fusion proteins of the murine c-Src SH3 domain, the human GRB2 SH2 and individual SH3 domains, and the human NCK SH2 domain were created using PCR to amplify the appropriate sequences from cDNA templates, cloned into pGEXKT, expressed and purified as described for Fig. 1. *In vitro* binding was assayed as for Fig. 1. SDS-boiled lysates were prepared as described¹⁵. Enhanced chemiluminescent exposure times: *a*, 1 min; *b*, 2 min; *c–g*, 30 s.



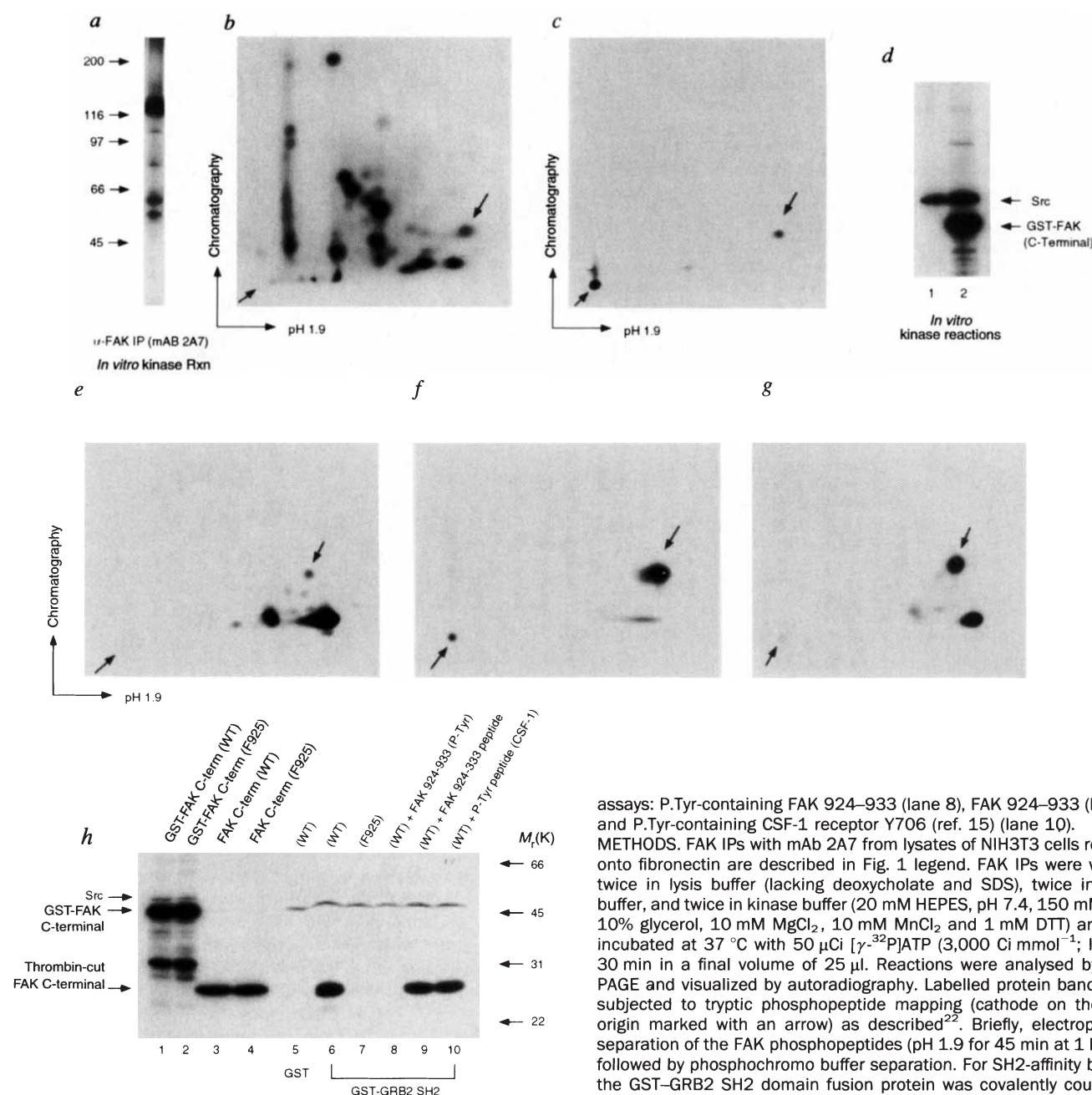


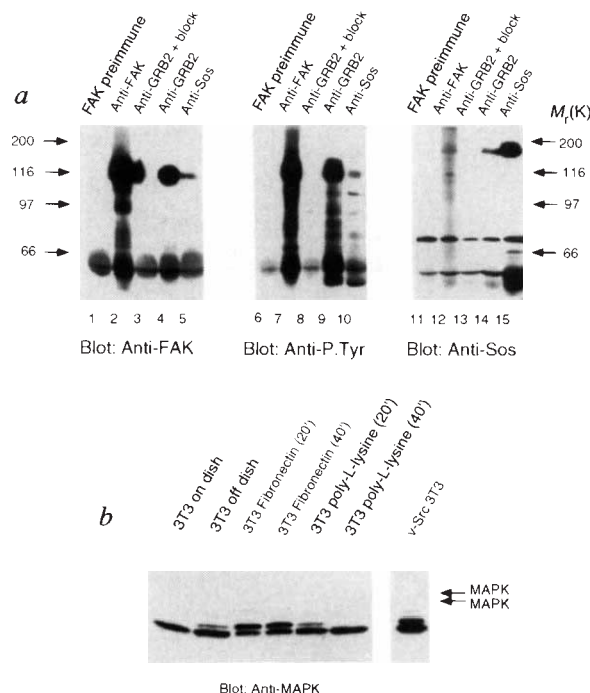
FIG. 4 Direct GRB2 SH2 binding to a P.Tyr-containing FAK tryptic phosphopeptide; FAK C-terminal domain phosphorylation by Src *in vitro*; mutation of FAK Tyr 925 blocks GRB2 SH2-mediated binding. **a**, A FAK IP was ³²P-labelled by an immune complex kinase reaction. **b**, The 116K ³²P-labelled FAK band was subjected to tryptic phosphopeptide mapping. **c**, An aliquot of the labelled tryptic-digested FAK peptides was incubated with the GRB2 SH2 domain and the bound material was resolved on a phosphopeptide map. **d**, Baculovirus-purified c-Src (lane 1) was used to phosphorylate 2.5 µg of the GST FAK C-terminal fusion protein (lane 2). **e**, Phosphorylated GST-FAK was subjected to tryptic phosphopeptide mapping. **f**, An aliquot of the labelled tryptic-digested GST-FAK was incubated with the GRB2 SH2 domain and the bound material was analysed by phosphopeptide mapping. **g**, Mix of samples shown in **e** and **f**. Sample origin and the GRB2 SH2 bound peptide locations are marked with arrows and the directions of electrophoresis and chromatography are shown. **h**, Baculovirus-purified c-Src phosphorylation of wild type (WT) (lane 1) or F925 (lane 2) GST-FAK C-terminal domain fusion proteins. Thrombin-cleaved phosphorylated WT (lane 3) or F925 (lane 4) FAK C-terminal domain (Gly 853 end). *In vitro* binding was assayed using GST (lane 5) or the GST-GRB2 SH2 domain (lanes 6–10) with thrombin-cut WT FAK C terminus (lanes 5, 6, 8–10) or with F925 FAK C terminus (lane 7). Peptide addition to *in vitro* binding

assays: P.Tyr-containing FAK 924–933 (lane 8), FAK 924–933 (lane 9), and P.Tyr-containing CSF-1 receptor Y706 (ref. 15) (lane 10).

METHODS. FAK IPs with mAb 2A7 from lysates of NIH3T3 cells replated onto fibronectin are described in Fig. 1 legend. FAK IPs were washed twice in lysis buffer (lacking deoxycholate and SDS), twice in HNTG buffer, and twice in kinase buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 10 mM MgCl₂, 10 mM MnCl₂ and 1 mM DTT) and then incubated at 37 °C with 50 µCi [³²P]ATP (3,000 Ci mmol⁻¹; ICN) for 30 min in a final volume of 25 µl. Reactions were analysed by SDS-PAGE and visualized by autoradiography. Labelled protein bands were subjected to tryptic phosphopeptide mapping (cathode on the right; origin marked with an arrow) as described²². Briefly, electrophoretic separation of the FAK phosphopeptides (pH 1.9 for 45 min at 1 kV) was followed by phosphochromatography. For SH2-affinity binding, the GST-GRB2 SH2 domain fusion protein was covalently coupled to glutathione-agarose as described for antibody coupling in Fig. 2 legend. The trypsin-digested FAK peptides were incubated for 2 h at 4 °C with ~20 µg of the coupled GRB2 SH2 domain in 0.5 ml of 50 mM NH₄HCO₃ pH 8.0. Bound material was pelleted by centrifugation, washed, and the peptides recovered by incubation in pH 1.9 buffer at 55 °C. The GST-FAK C-terminal domain fusion protein was expressed and purified as described for Fig. 1. FAK Tyr 925 was mutated to phenylalanine by recombinant PCR. Phosphorylation of the GST-FAK fusion proteins for the binding assays was done with 20 ng baculovirus c-Src in the presence of 10 µM ATP (10 µCi nmol⁻¹ [³²P]ATP) in a standard kinase assay as described. Phosphorylated thrombin-cleaved FAK C-terminal domains were prepared by stopping c-Src phosphorylation reactions with EDTA (5 mM), binding fusion proteins to glutathione-agarose, washing, and recovering the FAK C-terminal fragment by thrombin-cleavage. For the GST-GRB2 SH2-binding assay, excess thrombin was inhibited by PMSF (1 mM), benzamide (1 mM) and aprotinin (10 µg ml⁻¹), and the mixture diluted into cell lysis buffer containing Triton. About 1 × 10⁵ c.p.m. of labelled WT or F925 FAK C-terminal domains in 1 ml were incubated for 2 h at 4 °C with 10 µg GST-GRB2 SH2 domain. Peptides were purified by HPLC chromatography, analysed by mass spectroscopy, and used at 100 µM in *in vitro* binding assays. The GRB2 bound FAK C-terminal domain was washed twice, analysed by SDS/15% PAGE and visualized by autoradiography.

FIG. 5 FAK associates with Sos in v-Src 3T3 cells and MAPK is activated by NIH3T3 cell adhesion to fibronectin. **a**, IPs from v-Src 3T3 cells with anti-FAK pre-immune serum control (lanes 1, 6 and 11), affinity-purified anti-FAK antibodies (lanes 2, 7 and 12), anti-GRB2 antibodies in the presence of excess peptide 'block' used to raise the GRB2 antiserum (lanes 3, 8 and 13), anti-GRB2 antibodies (lanes 4, 9 and 14), and anti-Sos antibodies (lanes 5, 10 and 15). Anti-FAK blot (lanes 1–5), anti-P.Tyr blot (lanes 6–10) and anti-Sos blot (lanes 11–15) of the IPs as resolved by SDS-PAGE. **b**, Anti-MAPK blot analysis of whole-cell lysates from serum-starved NIH3T3 cells grown on plastic (lane 1), detached from plastic (lane 2), detached from plastic and replated on fibronectin for 20 min (lane 3) or 40 min (lane 4) and detached from plastic and replated on poly-L-lysine for 20 min (lane 5) or 40 min (lane 6). Whole-cell lysate (50 μ g total protein) from proliferating v-Src 3T3 cells (lane 7). MAPK* indicates the phosphorylated form of ERK2.

METHODS. IPs from v-Src transformed 3T3 cell lysates (1.5 mg total cell protein per point in 1 ml) were resolved by SDS/10% PAGE and immunoblotted with anti-FAK or anti-P.Tyr antibodies as described for Fig. 1. Polyclonal anti-Sos (C-20 peptide antibody) (Santa Cruz Biotechnology) was used at 0.5 μ g ml⁻¹ for immunoblotting. Enhanced chemiluminescent exposure times in **b** were 2.5 min. NIH3T3 whole cell lysates (50 μ g total protein) were analysed for MAPK activation by immunoblotting using anti-MAPK (mAb B3B9) at 1 μ g ml⁻¹. Enhanced chemiluminescent exposure time in **c** was 10 s.



To characterize the GRB2/FAK interaction, v-Src 3T3 lysates were incubated with GST fusion proteins containing full-length GRB2, the GRB2 SH2 domain, individual GRB2 SH3 domains, and full-length GRB2 with an R86K SH2-domain-inactivating mutation¹². Only full-length GRB2 and the GRB2 SH2 bound to FAK (Fig. 3g). No FAK binding was detected with full-length R86K GRB2 or with either of the GRB2 SH3 domains alone. Evidence for direct binding of GRB2 and the GRB2 SH2 to FAK was obtained using SDS-denatured v-Src NIH-3T3 lysates (Fig. 3g, lanes 7 and 8).

To identify the GRB2 SH2-binding site on FAK, FAK IPs from fibronectin-replated NIH3T3 cells were labelled *in vitro* with [γ -³²P]ATP (Fig. 4a). Gel-purified FAK was subjected to tryptic phosphopeptide mapping and phosphoamino-acid analysis. FAK phosphorylated *in vitro* contained only P.Tyr (data not shown) and yielded a phosphopeptide map with >10 P.Tyr-containing peptides (Fig. 4b). Tryptic phosphopeptide maps of *in vivo* labelled FAK from v-Src 3T3 cells were similar to those of *in vitro* labelled FAK, but had extra phosphoserine-containing peptides¹³ (data not shown). The ~60K band in the FAK IP (Fig. 4a) was identified as c-Src by phosphopeptide mapping (data not shown).

To test whether the GRB2 SH2 binds to a specific phosphorylated tyrosine on FAK, GST-GRB2 SH2 was incubated with a tryptic digest of *in vitro* phosphorylated FAK (Fig. 4b). Bound peptides were analysed by phosphopeptide mapping. GST-GRB2 SH2 specifically bound one peptide out of the many in the total FAK digest (Fig. 4b, and c, arrows). As a control, we used the NCK, phospholipase C γ and p85 SH2 domains in similar analyses; all bound different FAK peptides from GST-GRB2 SH2 (data not shown).

FAK contains a GRB2 SH2 consensus binding site at Tyr 925 (YENV)¹⁴. To test whether GRB2 binds to this site, a GST fusion protein containing the FAK carboxy terminus (Gly 853 end) was phosphorylated with purified c-Src (Fig. 4d) and digested with trypsin (Fig. 4e). Out of the total digest, GST-GRB2 SH2 selectively bound one peptide (Fig. 4f, g) which comigrated with the GRB2 SH2 bound peptide from immunoprecipitated FAK (data not shown).

To determine whether Tyr 925 of FAK is essential for GRB2 SH2 binding, Tyr 925 was mutated to phenylalanine in the GST-FAK C-terminal protein. The wild type and F925 FAK GST fusion proteins were phosphorylated in solution with purified c-Src (Fig. 4h, lanes 1 and 2), bound to glutathione-agarose, washed, and the FAK fragment released from GST by thrombin cleavage (Fig. 4h, lanes 3 and 4). GST-GRB2 SH2 bound wild-type FAK (Fig. 4h, lane 6) but not F925 FAK (Fig. 4h, lane 7). A synthetic phosphopeptide corresponding to FAK 924–933 blocked GRB2 SH2 binding to wild-type FAK (Fig. 4h, lane 8), whereas the unphosphorylated FAK 924–933 peptide (Fig. 4h, lane 9) and an irrelevant P.Tyr-containing CSF1-receptor peptide¹⁵ (Fig. 4h, lane 10) did not. Phosphopeptide mapping of phosphorylated F925 FAK showed that only the GRB2 SH2-bound phosphopeptide from wild-type FAK (Fig. 4e) was absent (data not shown). These results show that P.Tyr 925 is the *in vitro* GRB2 SH2-binding site on FAK.

As GRB2 mediates signal transduction from growth factor receptor PTKs to the Ras/MAPK pathway through interaction with the Ras GDP/GTP exchange protein Sos^{12,16,17}, we tested whether Sos was present in FAK IPs from v-Src 3T3 lysates and vice versa. FAK was detected in Sos IPs by anti-FAK (Fig. 5a, lanes 5) and by anti-P.Tyr blotting (Fig. 5a, lane 10). Conversely, Sos was detected in FAK IPs by anti-Sos blotting (Fig. 5a, lanes 12). As binding of Sos to GRB2 is mediated by the GRB2 SH3 domains¹⁷, we speculate that this FAK/Sos interaction is indirectly mediated by an SH2/SH3 adaptor protein such as GRB2 or NCK.

To obtain further evidence that integrin-mediated signal transduction is coupled to activation of the Ras pathway, we tested whether plating NIH3T3 cells onto fibronectin causes MAPK activation¹⁸. In quiescent NIH3T3 cells, a single MAPK band was detected with anti-ERK2 monoclonal antibody (Fig. 5b, lane 1). Cell detachment resulted in slight activation of MAPK (Fig. 5b, lane 2), as evidenced by the appearance of an ERK2 band with decreased SDS-PAGE mobility. This slight MAPK activation, possibly due to nonspecific trypsin-mediated events, returned to baseline by 40 min after replating onto poly-L-lysine (Fig. 5b, lane 6). In contrast, replating onto fibronectin resulted

in strong MAPK activation within 20 min (Fig. 5b, lane 3), which was sustained up to 40 min (Fig. 5b, lane 4). This fibronectin-mediated MAPK activation result agrees with a report that integrin-mediated cell adhesion causes activation and nuclear translocation of MAPKs¹⁹.

Our findings that fibronectin stimulation of NIH3T3 cells promotes FAK tyrosine phosphorylation, c-Src and GRB2 association with FAK, and MAPK activation, complements and extends earlier findings that $\alpha_2\beta_1$ integrin activation in T lymphoblasts promotes accumulation of Ras-GTP²⁰. Based on our results, we propose the following integrin-stimulated signal transduction model. Upon integrin-mediated cellular binding to fibronectin, FAK autophosphorylation is stimulated and phosphorylated Tyr 397 is exposed, leading to the SH2-mediated binding of c-Src or related PTKs to FAK. Subsequent phosphorylation of FAK by Src-related PTKs creates binding sites for other SH2 proteins such as GRB2, NCK and phosphatidylinositol-3-OH kinase. In particular, GRB2 binding to FAK at Tyr 925 may lead to the formation of multiprotein signalling complexes which promote activation of the Ras signal transduction pathway. In v-Src 3T3 cells, this pathway may be activated in the absence of integrin stimulation owing to the constitutive association of v-Src and FAK.

Our results indicate that signal transduction events are elicited by integrin-mediated FAK phosphorylation that are similar to those stimulated by growth-factor binding to receptor PTKs. The fact that fibronectin-deficient mice fail to develop as a result of mesodermal fibroblast abnormalities²¹ indicates that fibronectin-mediated signalling through FAK activation is critical in development. □

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Heterogeneity of channel density in inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores

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INOSITOL-1,4,5-trisphosphate (InsP_3)-induced Ca^{2+} release is a key mechanism for intracellular Ca^{2+} mobilization¹. The rate of Ca^{2+} release declines progressively with time until a higher concentration of InsP_3 is added, which is referred to as the incremental detection mechanism². Two hypotheses have been postulated to explain these complex kinetics: (1) Ca^{2+} stores consist of multiple compartments (quanta) with different sensitivities to InsP_3 (refs 3–7), and (2) the rate of Ca^{2+} release is modulated by the Ca^{2+} concentration in the lumen of Ca^{2+} stores^{8–12}. We studied this phenomenon by real-time measurement of the luminal Ca^{2+} concentration of Ca^{2+} stores using a Ca^{2+} -sensitive fluorescent dye, but our results were not explained by either of these hypotheses. Here we report that the complex kinetics of Ca^{2+} release results from the heterogeneous density of equally InsP_3 -sensitive channels on the Ca^{2+} stores. This heterogeneity creates Ca^{2+} stores with apparently different sensitivities to InsP_3 , which may have different functions in Ca^{2+} mobilization.

We loaded a low-affinity Ca^{2+} -sensitive fluorescent dye, Fura-2/AM¹³, inside the Ca^{2+} stores in permeabilized vascular smooth muscle cells. Figure 1a shows that activation of Ca^{2+} pumps induced opposite changes in fluorescence intensity with 325 and 375 nm excitation. Subsequent addition of 10 μM InsP_3

reversed the fluorescence changes. The ratio of the two signals (F_{325}/F_{375}) gives the luminal Ca^{2+} concentration ($[\text{Ca}^{2+}]_L$) within InsP_3 -sensitive Ca^{2+} stores because (1) the rate of increase of the ratio during Ca^{2+} loading depends on the Ca^{2+} concentration in the presence of Mg-ATP, which is not increased with either Ca^{2+} or Mg²⁺ alone; (2) the change in the ratio is suppressed in the presence of cyclopiazonic acid, an inhibitor of Ca^{2+} -ATPase¹⁴ (Fig. 1b); (3) the InsP_3 -induced decline is blocked by heparin, an inhibitor of InsP_3 -induced Ca^{2+} release¹⁵ (Fig. 1c).

In the presence of Mg-ATP and a low concentration of EGTA, the submaximal InsP_3 -induced Ca^{2+} release took place in two phases: the initial rapid and subsequent slow phases (Fig. 2a), as shown previously^{2,3,5,8,10,12}. However, Ca^{2+} -mediated feedback regulation of the Ca^{2+} release^{16,20} and simultaneous Ca^{2+} uptake may interfere with the observed rate of Ca^{2+} release. The major advantage of our method is that we are able to observe Ca^{2+} release in the presence of a strong Ca^{2+} buffer. We therefore analysed the time course of Ca^{2+} release at a constant cytoplasmic Ca^{2+} concentration in the absence of Mg-ATP. Even under such conditions the rate of Ca^{2+} release at 30 nM InsP_3 declined gradually until a higher concentration of InsP_3 was added; the time course deviated from a single exponential (Fig. 2b), but this could not have been a result of saturation of Fura-2/AM, which would create a deflection in the opposite direction.

It could be argued that the decline of the Ca^{2+} release rate is caused by Ca^{2+} -dependent inactivation of the channel^{16–21} due to local build-up of Ca^{2+} concentration near the channel pore. But the decline of rate with time was similar with 10 mM BAPTA, a faster Ca^{2+} buffer (Fig. 2c, trace 1). Furthermore, interruption of InsP_3 application did not restore the Ca^{2+} release rate (Fig. 2c, trace 2), although restoration would be expected if the decline was due to inactivation of the channel. Only when the store was reloaded during the removal of InsP_3 was the

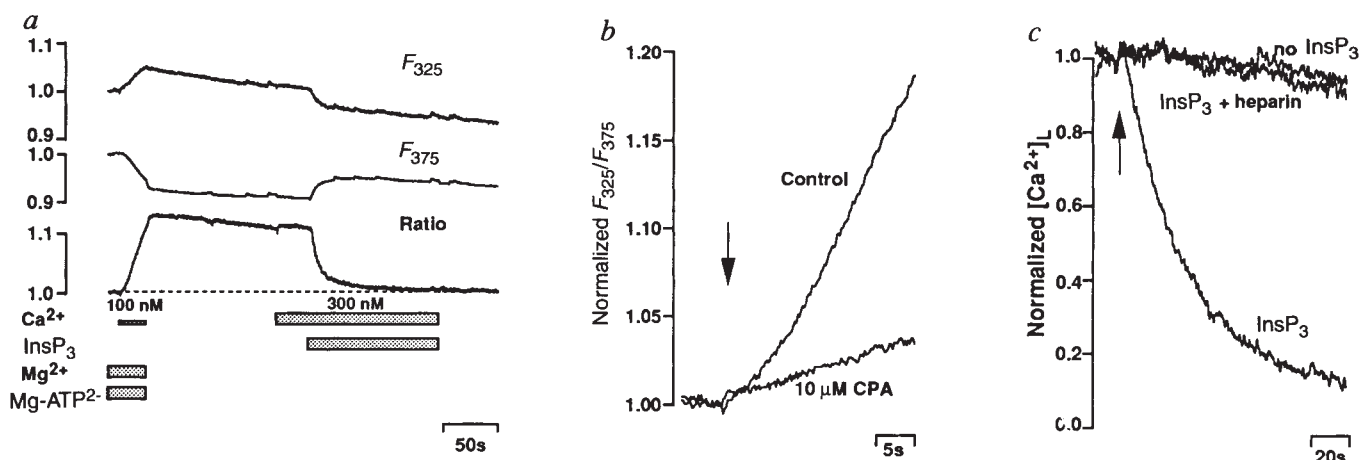
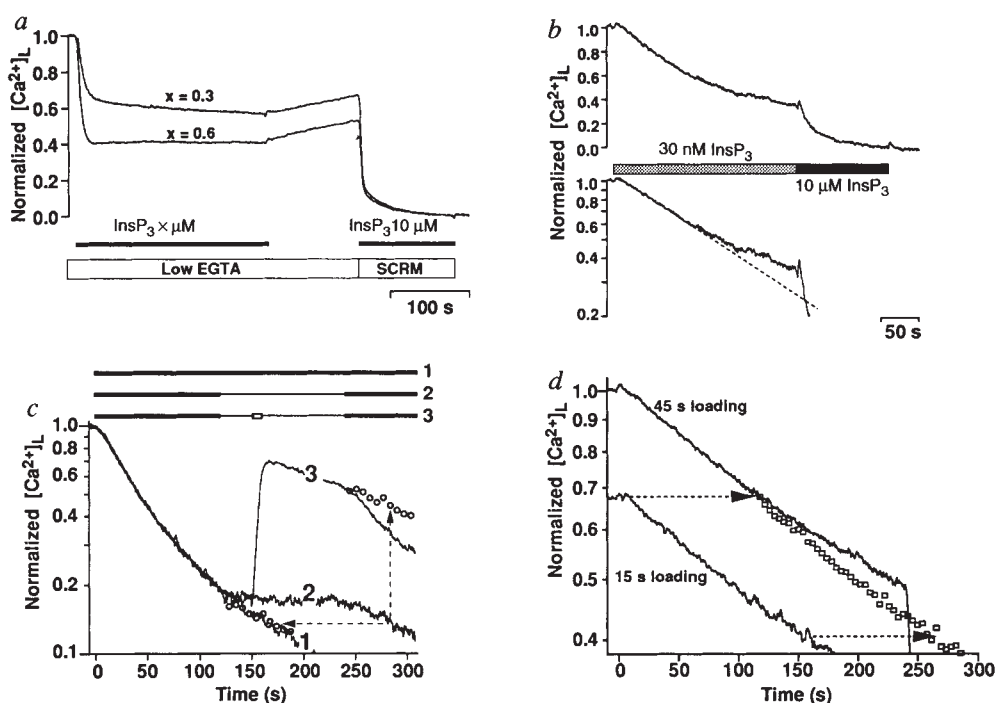


FIG. 1 Changes in the luminal Ca^{2+} concentration of Ca^{2+} stores during Ca^{2+} uptake and release measured with intraluminally loaded Fura2. **a**, Upper and middle traces: fluorescence intensities with excitation wavelengths of 325 nm (F_{325}) and 375 nm (F_{375}), respectively. Lower trace: ratio of F_{325} to F_{375} . Each value was normalized using its prestimulation value. Solutions were changed as indicated under the traces and were continuously perfused. **b**, Inhibitory effect of cyclopiazonic acid (CPA) on Ca^{2+} uptake. Ca^{2+} (100 nM) was applied at the point indicated by the arrow, with or without 10 μM CPA. **c**, Inhibition by heparin (100 $\mu g\ ml^{-1}$) of Ca^{2+} release induced by 100 nM $InsP_3$ applied at the point indicated by the arrow. Normalization of $[Ca^{2+}]_L$ was such that 1 and 0 correspond, respectively, to the Ca^{2+} loaded level and the level after depletion by 10 μM $InsP_3$. The depleted level was the same after addition of 100 μM

$InsP_3$. Data in this and other figures were representative of 4 or 5 experiments unless stated otherwise.

METHODS. Thin smooth muscle bundles (200–250 μm wide, 60–80 μm thick, 3 mm long) were dissected from the portal vein of guinea-pigs and were incubated for 3.5–5 h at 35 $^{\circ}C$ in physiological salt solution containing 20–40 μM Fura2-AM and 0.005% Pluronic-F127. The Fura2-loaded bundles were then permeabilized in a relaxing solution containing 50 $\mu g\ ml^{-1}$ saponin or 40 μM β -escin for 30–60 min. Fluorescence intensity of Fura2 remaining after the permeabilization²⁶ was measured at >500 nm with 325 and 375 nm excitation alternating at 128 Hz. The experimental set-up and solutions have been described^{16,20}. Standard Ca^{2+} -releasing medium contained no $Mg-ATP$ and 300 nM Ca^{2+} (10 mM EGTA). Experiments were done at room temperature (21–23 $^{\circ}C$).

FIG. 2 No involvement of inactivation and luminal Ca^{2+} dependence in time-dependent decline in $InsP_3$ -induced Ca^{2+} release rate. **a**, 'Quantal' Ca^{2+} release at submaximal concentrations of $InsP_3$ (horizontal bar) in the presence of 0.5 mM $Mg-ATP$ and 10 μM EGTA. The creeping up after washout of $InsP_3$ indicates a Ca^{2+} uptake rate that has been balancing the $InsP_3$ -induced Ca^{2+} release. The Ca^{2+} store was then depleted by 10 μM $InsP_3$ in standard Ca^{2+} releasing medium (SCRM). **b**, Time course of $InsP_3$ -induced Ca^{2+} release in SCRM. Data are displayed in a linear (top) or semi-logarithmic (lower panel) plot. **c**, Effect of the interruption of $InsP_3$ application on subsequent Ca^{2+} release. Ca^{2+} concentration was buffered by 10 mM BAPTA at 300 nM throughout the experiment. Ca^{2+} was released by 400 nM $InsP_3$ (thick lines) without (traces 1) and with (traces 2 and 3) the interruption of $InsP_3$ application (thin lines). In trace 3, the Ca^{2+} store was loaded by the 8-s application of $Mg-ATP$ (10 μM) (box). Circles represent the second Ca^{2+} release of trace 2 shifted horizontally and vertically for comparison. **d**, Effect of the extent of Ca^{2+} loading on the rate of $InsP_3$ (30 nM)-induced Ca^{2+} release. Ca^{2+} stores were loaded with 300 nM Ca^{2+} for either 15 or 45 s. The curve of Ca^{2+} release following 15-s Ca^{2+} loading was shifted to the right for comparison with the rate of Ca^{2+} release at the same Ca^{2+} loading (squares). The average rate



of Ca^{2+} release with partial loading ($5.83 \pm 0.67 \times 10^{-3} s^{-1}$) was higher than that after partial depletion ($3.87 \pm 0.37 \times 10^{-3} s^{-1}$; $n = 7$, $P < 0.01$, paired t-test). The extent of loading was estimated as described²⁷. The level of Ca^{2+} loading for 45 s corresponded to about a quarter of quasi-physiological Ca^{2+} loading at a cytosolic Ca^{2+} concentration of 100 nM.

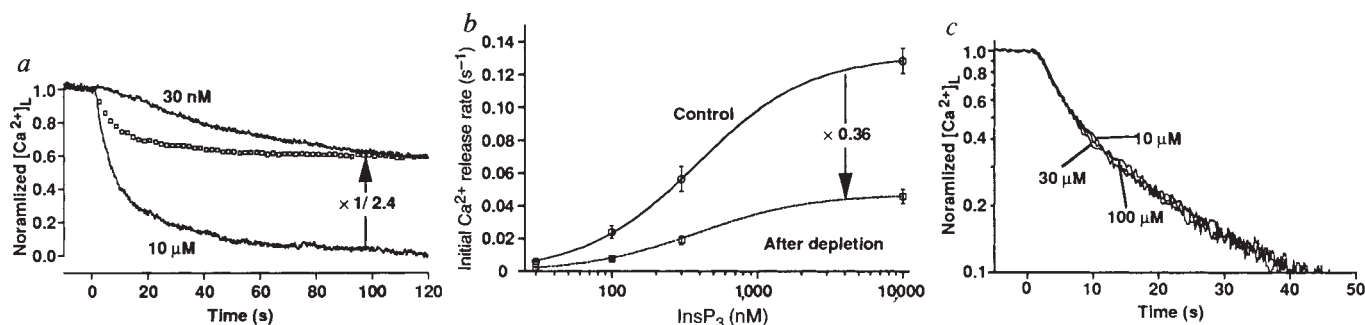


FIG. 3 Dependence of Ca^{2+} release rate on the concentration of InsP_3 . **a**, Ca^{2+} release was induced by either 30 nM (upper trace) or 10 μM (lower trace) InsP_3 . The trace at 10 μM is compressed vertically by a factor of 2.4 to give the sequence of squares for time course comparison. **b**, Effect of partial depletion of the store on the InsP_3 sensitivity of Ca^{2+} release. The initial rate of Ca^{2+} release was determined by fitting a single exponential to the data within 30% reduction from the initial $[\text{Ca}^{2+}]_i$ and was plotted against the concentrations of InsP_3 with or without prior exposure to 30 nM InsP_3 for 240 s (mean $\pm$ s.e.m., $n=5-6$). The continuous curve (Hill's equation) was fitted to the control data by a nonlinear least-square method. The dotted curve represents the

fitted curve to the control data scaled down by 0.36, which fits the data well after depletion. **c**, Semi-logarithmic plots of time courses of Ca^{2+} release at 10, 30 and 100 μM InsP_3 . These are superimposable, indicating that maximal activation is attained at these concentrations. In our freshly dissected smooth muscle tissue there is probably no significant fragmentation of the Ca^{2+} store during permeabilization²² because we obtained the same non-exponential time course of Ca^{2+} release in (1) cells mildly permeabilized with α -toxin, whose effect is restricted to the plasma membrane²⁸, or (2) cells treated with 20 μM GTP and 4% polyethylene glycol, a procedure used to fuse fragmented vesicles²⁹.

release rate restored (Fig. 2c, trace 3). Reloading was essential as neither Mg-ATP alone nor Ca^{2+} and Mg-ATP with cyclopiazonic acid were able to induce recovery. These observations suggest that inactivation mechanisms are not involved in the decline of Ca^{2+} release rate.

We then asked whether the decline in the luminal Ca^{2+} concentration attenuated the rate of Ca^{2+} release^{8,12}. The rate of Ca^{2+} release with partial loading was higher than that after partial depletion subsequent to standard loading, even though the average Ca^{2+} content was the same (Fig. 2d). Thus the luminal Ca^{2+} concentration is unlikely to be a main factor determining the time-dependent decline in the Ca^{2+} release rate.

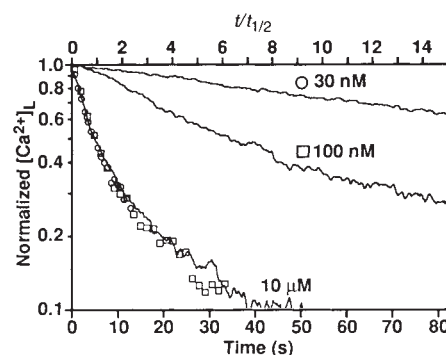
Our results could not be reconciled with Ca^{2+} release from a single uniform Ca^{2+} store, so, heterogeneous multicompartiment models were considered. One such model proposes that Ca^{2+} release is quantal: each compartment responds in an all-or-none fashion with different sensitivity to InsP_3 (refs 3-7). Such a mechanism predicts that: (1) the scaled time course of Ca^{2+} release should be independent of InsP_3 concentration because the rate of release is proportional to the number of quanta that respond to a given concentration of InsP_3 ; (2) if stores were depleted with low concentrations of InsP_3 , the remaining stores

should respond only to higher concentrations of InsP_3 , so InsP_3 sensitivity would be decreased; (3) supramaximal concentrations of InsP_3 should elicit monotonical Ca^{2+} release because all quanta would be homogeneously activated.

In Fig. 3a we compare the time courses of Ca^{2+} release by 30 nM and 10 μM InsP_3 . Scaling of the two records shows that they are quite different, disagreeing with the first prediction. We then analysed responsiveness to InsP_3 with and without prior partial depletion by 30 nM InsP_3 . The rates of Ca^{2+} release at all concentrations of InsP_3 were proportionally reduced by ~65% after partial depletion (Fig. 3b). Thus there is no change in the InsP_3 sensitivity, which contradicts the second prediction. To evaluate the third point, we analysed the time courses of Ca^{2+} release at supramaximal concentrations of InsP_3 (10, 30 and 100 μM) (Fig. 3c). They showed clear deviation from a single exponential at variance with the third prediction.

Our results indicate that the decline of Ca^{2+} release rate is unlikely to be the result of quantal Ca^{2+} release. Ca^{2+} release rate is proportional to channel activity multiplied by channel density. Thus the alternative heterogeneous compartment model is that there are compartments with different densities of InsP_3 receptors. In this model, the time course of Ca^{2+} release is

FIG. 4 Normalized time course of Ca^{2+} release at different InsP_3 concentrations. Continuous lines: time courses of Ca^{2+} release at 30, 100 nM and 10 μM InsP_3 (lower abscissa). Ca^{2+} release was observed for up to 360 s at the lower InsP_3 concentrations. Time course at 30 nM (circles), 100 nM (squares) and 10 μM InsP_3 (continuous line) was normalized to the half-time ($t_{1/2}$) of Ca^{2+} release for each InsP_3 concentration (upper abscissa) to examine whether Ca^{2+} stores are homogeneously sensitive to InsP_3 on the basis of the following consideration. If we assume the Ca^{2+} store is composed of two compartments that release Ca^{2+} exponentially with p -times corresponding to different rate constants (r and pr), then the total Ca^{2+} concentration (x) at time t is expressed as $x = a_1 \cdot e^{-rt} + a_2 \cdot e^{-prt}$, where a_1 and a_2 represent the initial Ca^{2+} content. Introducing dimensionless parameters $T = t/t_{1/2}$ (normalized time) and $R = rt_{1/2}$, we can rewrite the equation as $x = a_1 \cdot e^{-RT} + a_2 \cdot e^{-pRT}$ (normalized time course). When $T = 1$, $a_1 \cdot e^{-R} + a_2 \cdot e^{-pR} = (a_1 + a_2)/2$. Thus R is a function of p and is independent of r . This means that the normalized time course depends only on p and T . The argument can be expanded to cases involving three or more compartments. Therefore it is only when p is independent of InsP_3 concentration (that is, InsP_3 sensitivity is the same in all compartments) that the normalized time courses are superimposable at all concentrations of InsP_3 .



independent of InsP_3 concentration if normalized to the half-time ($t_{1/2}$) (Fig. 4 legend). This prediction does not hold if there are compartments with different InsP_3 sensitivities. In Fig. 4 we compare the time courses of Ca^{2+} release at 30, 100 nM and 10 μM InsP_3 . When normalized with $t_{1/2}$, they become superimposable, indicating that the results are consistent with the present model. We found that two exponential components were sufficient to fit the time course of Ca^{2+} release reasonably well, and our results are consistent with the faster component having $65.2 \pm 9.7\%$ of the total capacity and 6.76 ± 2.60 times greater channel density (mean $\pm$ s.d., $n = 52$). Our results may also be consistent with the compartments having different types of InsP_3 receptors which have the same affinity for InsP_3 but have a different open probability or single-channel conductance. The Ca^{2+} stores may be structurally continuous^{22,23} but they can be functionally divided if diffusion of Ca^{2+} within the Ca^{2+} stores is restricted as a result of a tortuous diffusion path and/or high-density intraluminal Ca^{2+} binding sites.

It has been suggested that there is regional heterogeneity of InsP_3 sensitivity in pancreatic acini cells²⁴ and in cultured smooth muscle cells²⁵. Although diverse InsP_3 sensitivity of Ca^{2+} stores has been attributed to the different types of InsP_3 receptors or to their different degrees of modification^{1,6}, this remains to be proved. On the other hand, heterogeneous density of InsP_3 receptors on Ca^{2+} stores may explain apparent variations in InsP_3 sensitivity, which may be amplified through regulation of InsP_3 receptors by cytoplasmic Ca^{2+} (refs 16–20) as Ca^{2+} -mediated sensitization of Ca^{2+} release may occur more easily in Ca^{2+} stores with higher densities of InsP_3 receptors. Thus stores with different densities of InsP_3 receptors may play different roles in

the generation of Ca^{2+} oscillations and Ca^{2+} waves. □

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Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun

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THE stress-activated protein kinases (SAPKs), which are distantly related to the MAP kinases, are the dominant c-Jun amino-terminal protein kinases activated in response to a variety of cellular stresses, including treatment with tumour-necrosis factor- α and interleukin- β (refs 1, 2). SAPK phosphorylation of c-Jun probably activates the c-Jun transactivation function³. SAPKs are part of a signal transduction cascade related to, but distinct from, the MAPK pathway¹. We have now identified a novel protein kinase,

called SAPK/ERK kinase-1 (SEK1), which is structurally related to the MAP kinase kinases (MEKs)⁴. SEK1 is a potent activator of the SAPKs *in vitro* and *in vivo*. An inactive SEK1 mutant blocks SAPK activation by extracellular stimuli without interfering with the MAPK pathway. Although alternative mechanisms of SAPK activation may exist, as an immediate upstream activator of the SAPKs, SEK1 further defines a signalling cascade that couples cellular stress agonists to the c-Jun transcription factor.

We previously isolated two *Xenopus* complementary DNAs, XMEK2 and XMEK3, that encode potentially novel MEKs⁵. XMEK2 is sufficiently different from MEK1 in structure to suggest it has a distinct role in signal transduction⁵. To define its function in mammalian cells, we isolated a murine homologue of XMEK2, which we refer to as SEK1 (Fig. 1a). A comparison of SEK1 with other mammalian and *Drosophila* MEKs and related yeast kinases demonstrates the extensive homology of the family (Fig. 1b) (reviewed in ref. 6). Specifically, subdomain VIII of the catalytic domain⁷ contains two sites of potential phosphorylation (at Ser 220 and Thr 224) which align with those in MEK1, whose phosphorylation by Raf-1 and MEK kinase results in MEK1 activation^{8–10}.

Northern blot analysis demonstrates that SEK1 messenger RNA is ubiquitously expressed at very high levels in brain and muscle (Fig. 1c), consistent with our previous findings of the XMEK2 pattern of expression during *Xenopus* development⁵.

To analyse SEK1 function, SEK1 was cloned into the mammalian expression vector pEBG, which drives the expression of a glutathione-S-transferase (GST) fusion protein, including the entire coding sequence of SEK1. pEBG-SEK1 was transfected into 293 cells and the cells were then treated with tumour-necrosis factor- α (TNF- α) or anisomycin, agonists known to activate the SAPKs *in vivo*¹, or epidermal growth factor (EGF), an agonist that does not activate SAPKs in most cell types¹. GST-SEK1, immobilized on glutathione-agarose, was assayed for its ability to activate recombinant prokaryotically expressed

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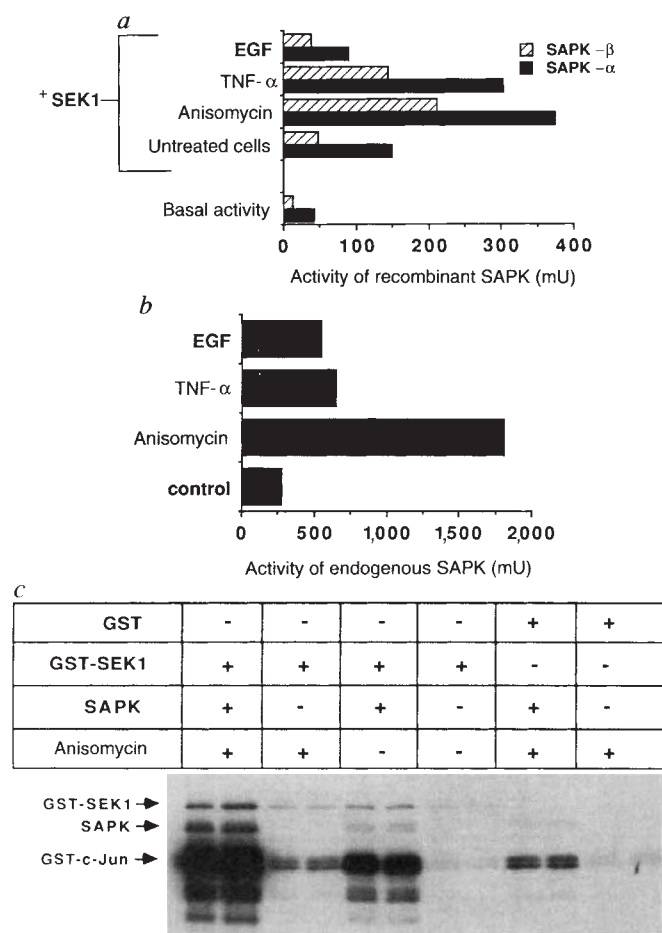


FIG. 2 Characterization of SEK1 function. **a**, SEK1 activates SAPK- α and SAPK-p54 β . GST-SEK1 was recovered from transfected 293 cells after treatment of the cells with the ligands shown and assayed for activation of recombinant SAPK isoforms. Values shown are for activated SAPK activity. **b**, Activity of endogenous SAPKs immunoprecipitated from non-transfected cells treated as in **a**; SAPK activation parallels activation of transfected SEK1. **c**, SEK1-catalysed phosphorylation of the SAPK polypeptide accompanies SAPK activation by SEK1. Recombinant SAPK- α or vehicle was incubated with GST only, GST-SEK1 from control cells, or GST-SEK1 from anisomycin-treated cells as indicated. GST-c-Jun was then added to measure SAPK activity. GST-c-Jun, SAPK and GST-SEK1 polypeptides are indicated. **d**, Phosphoamino-acid analysis of SEK1 phosphorylation of SAPK. Left, top, SEK1 phosphorylation of wild-type SAPK- α . Right panel phosphorylation of SAPK- α (K→R) mutant. Bottom left, relative positions of phosphoamino acids: Y, tyrosine; T, threonine; S, serine.

contrast, EGF fails to stimulate SEK1 activity beyond the three-fold levels seen in unstimulated cells. Parallel assays of endogenous SAPK activity in 293 cells (Fig. 2b) demonstrates that the degree of activation of the SAPKs in response to TNF- α and anisomycin parallels closely that of the recombinant of GST-SEK1. There is, however, a modest activation of SAPK by EGF in 293 cells which is not reflected by SEK1 activation. This result implies that SEK-independent mechanisms of SAPK activation might exist.

MEKs are dual-specificity protein kinases which phosphorylate MAPK polypeptides at the tyrosine and threonine residues whose phosphorylation is required for activation¹¹. SAPKs also require Tyr and Thr phosphorylation for activity^{2,12}. Under conditions that result in *in vitro* SAPK activation by SEK1 (Fig. 2c), wild-type SAPK is phosphorylated at Tyr, Ser and Thr (Fig. 2d, top left), whereas a kinase-inactive SAPK mutant is phosphorylated predominantly at Tyr, with no detectable Thr phosphorylation (Fig. 2d, top right). Thus SEK1 does not show

METHODS. pEBG is a mammalian expression vector derived from pEF-BOS (ref. 24) by insertion of a polylinker containing *Bam*HI and *Cl* sites. Expression of a GST-insert fusion protein is driven by the EF-1 α promoter. SEK1 K→R was produced by an overlapping PCR method²⁵ using a mutant PCR primer (5'-CAGATAATGGCAGTTAGAAGAATTCCG-TCAACT-3') to produce an A-to-G transposition at nt 414, resulting in a Lys-to-Arg substitution at Lys 129. Prokaryotically expressed SAPK- α , p54 β and ERK1 were expressed from the pGEX-KG vector, purified by glutathione-agarose chromatography²⁶. Transfection into 293 cells was by the calcium phosphate method; 5 μ g of the relevant plasmids were transfected. GST-SEK1, MEK1 or SAPK- β were isolated as follows. Cells were serum-starved for 16 h and then treated with anisomycin (50 μ g ml⁻¹ for 40 min, TNF- α (50 ng ml⁻¹, 15 min), EGF (100 ng ml⁻¹, 15 min) or PMA (1 μ M for 20 min to activate MEK1), at which time cells were lysed in 1 ml ice-cold lysis buffer¹. Lysates were cleared by centrifugation and incubated with glutathione-agarose for 30 min and washed as described¹. SAPKs were immunoprecipitated and assayed as before¹. For GST-SEK1 and GST-MEK1 assays, 30 μ l 1:1 beads (GST-SEK1 or MEK1) in kinase assay buffer¹ were incubated at 30 °C for 30 min with 15 μ l 0.15 mg ml⁻¹ SAPK or MAPK preparation and [γ -³²P]ATP (100 μ M, 15 μ Ci). At this time, 1 μ g GST-c-Jun (SAPK assays) or MBP (ERK1 assays) and an additional 100 μ M [γ -³²P]ATP were added and the reaction continued for 15 min. Proteins were separated by SDS-PAGE and the appropriate bands excised and counted. Background activity in the absence of SAPK/ERK1 was subtracted from the values in **a**: 25 mU (control), 75 mU (TNF- α) 75 mU (anisomycin) and 20 mU (EGF). Experiments were performed twice and assayed in duplicate; a representative experiment is shown. A unit of SAPK activity is defined in ref. 1. Phosphoamino-acid in SAPK were analysed as described²⁷.

prominent dual specificity and appears to phosphorylate SAPKs preferentially at Tyr. Insofar as SAPKs are proline-directed¹³ and the site of SAPK activating phosphorylation is TPY^{1,2}, a significant portion of the regulatory Thr phosphorylation could arise as a result of autophosphorylation, occurring after SEK1-catalysed Tyr phosphorylation. Indeed, the MAPK-specific MEKs show a similar preference for Tyr when presented with, as substrates, MAPK mutants which are incapable of phosphotransferase activity¹⁴.

To determine the specificity of SEK1, we compared the ability of SEK1 to activate SAPK- α and ERK1. 293 cells were transfected with either pEBG-SEK1 or pEBG-MEK1. Cells were treated with either anisomycin to activate SEK1, or with phorbol myristate acetate (PMA) to activate MEK1. As shown in Fig. 3a, SEK1 dramatically activates SAPKs under conditions that catalyse little or no activation of ERK1. GST-SEK1 activates ERK1 1.1-fold, compared with an 18-fold activation of SAPK- α . By contrast, incubation of ERK1 with GST-MEK1 beads

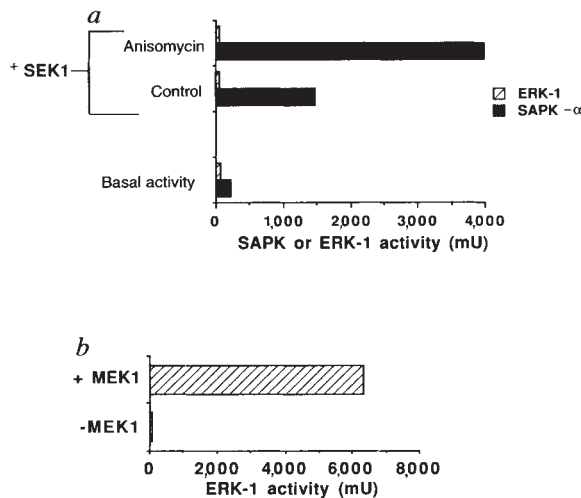
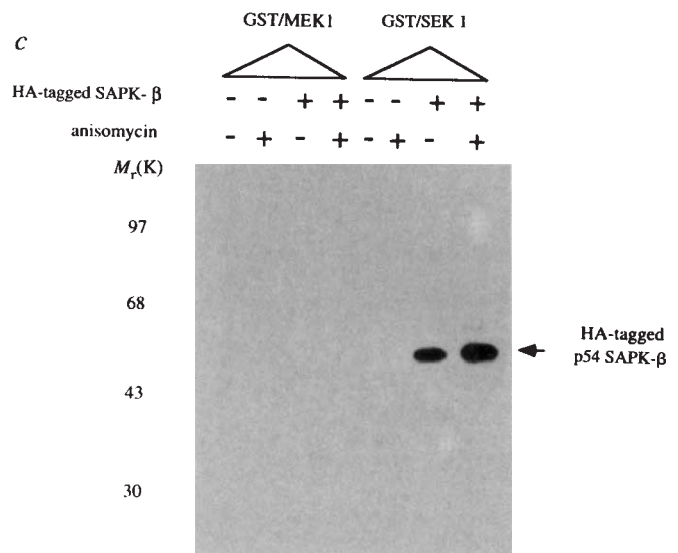


FIG. 3 Specificity of SEK1 for SAPKs. *a*, Comparison of SEK1 activity towards SAPK- α 1 and MAPK (ERK1). *b*, The ERK1 preparation can be activated by MEK1. *c*, Physical association between SAPK and SEK1. COS7 cells were transfected with pEBG-MEK1 (lanes 1, 2, 5 and 6) or SEK1 (lanes 3, 4, 7 and 8) and HA-SAPK-p54 β (lanes 5–8). Odd-number lanes, control cells; even-number lanes, anisomycin-treated cells. GST-MEK1 and SEK1 were recovered on glutathione-agarose and probed by immunoblotting with anti-HA antiserum.

gives >20-fold activation of the ERK1 MBP-kinase activity (Fig. 3*b*). Thus SEK1 shows at least 15-fold more activity towards SAPK than towards ERK1. On the other hand, MEK1 gives no detectable activation of SAPKs *in vitro* (refs 1, 2 and data not shown).

If SAPKs were specific SEK1 substrates, an association of these two polypeptides *in vivo* might be detectable. COS7 cells were cotransfected with haemagglutinin (HA)-tagged SAPK-p54 β and either pEBG-SEK1 or pEBG-MEK1. GST-SEK1 or GST-MEK1 were then purified from cell lysates by glutathione-agarose chromatography and immunoblotted with an antibody against the HA tag. As seen in Fig. 3*c*, HA-tagged SAPK complexes and copurifies specifically with GST-SEK1 and not GST-MEK1. We do not know if the association between SAPK and SEK1 is direct or is through a third, unidentified molecule.

Further evidence of the physiological significance of SEK1 as an upstream activator of the SAPKs is provided by the properties of a mutant kinase-inactive pEBG-SEK1 construct in which the critical lysine (Lys 129) in the ATP-binding site has been mutated to arginine. This mutant, pEBG-SEK1K-R, was



METHODS. GST-SEK1 and MEK1 were assayed as described in Fig. 2 legend. COS7 cells were transfected by the DEAE-dextran method²⁸. Immunoblotting was with the ECL system (Amersham). 1U ERK1 will transfer 1 pmol min⁻¹ PO₄ from ATP to MBP. As for Fig. 2, activity of contaminating c-Jun kinase or MBP kinase in the preparations of GST-SEK1 and MEK1 was subtracted from the values shown. These activities were 14 mU (control SEK1 c-Jun kinase), 135 mU (anisomycin SEK1 c-Jun kinase), and 82 mU (MEK1 MBP kinase).

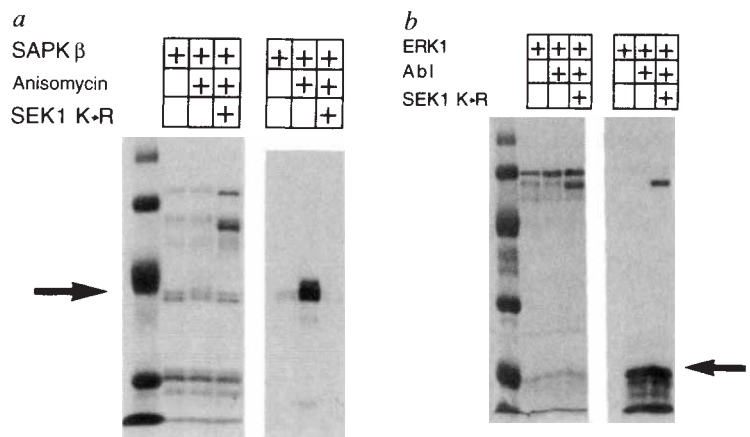
cotransfected with pEBG-SAPK-p54 β into 293 cells and the ability of anisomycin to activate GST-SAPK activity in these cells assayed. Anisomycin activates the GST-SAPK β (Fig. 4*a*, right; compare lanes 1 and 2). But coexpression of the GST-SAPK β with the mutant SEK1K-R suppresses this activation completely (Fig. 4*a*, right; compare lanes 2 and 3). Thus GST-SEK1K-R can act as a dominant inhibitor of SAPK activation *in vivo*, indicating that anisomycin activation of the SAPKs requires elements that interact with SEK1 and that these elements (for example, SEK kinases) can be completely sequestered by overexpression of recombinant inactive SEK1.

Evidence for the specificity of the SEK1 K $\rightarrow$ R mutant for the SAPK pathway is shown in Fig. 4*b*. Expression of the transforming *abl* gene leads to activation of p42/p44 MAPKs, presumably through the Ras pathway (refs 15 and 16, and Fig. 4*b*, right, lanes 1 and 2). The activation of the ERK1 by Abl is not interfered with by SEK1(K $\rightarrow$ R), whereas this mutant completely blocks SAPK activation (compare Fig. 4*a* and *b*, right panels).

Whereas several mechanisms of SAPK activation may exist, on the basis of the *in vitro* activation of SAPKs by SEK1, the

FIG. 4 Expression of a kinase-inactive SEK1, SEK1(K $\rightarrow$ R), inhibits anisomycin activation of SAPKs but does not interfere with Abl activation of MAPKs. *a*, Expression of SEK1(K $\rightarrow$ R) inhibits activation of SAPK by anisomycin. pEBG-SAPK-p54 β was expressed in 293 cells alone or together with pEBG-SEK1(K $\rightarrow$ R). Cells were treated with anisomycin and SAPK activity measured. Left, Coomassie-blue stained gel; right, autoradiogram. Arrow indicates GST-c-Jun. *b*, 293 cells were transfected with a retroviral vector encoding a SH3 deleted transforming Abl gene and pEBG-ERK1, or pEBG-ERK1 plus pEBG-SEK1(K $\rightarrow$ R). GST-ERK1 was isolated and assayed for MBP kinase activity. Left, Coomassie-blue stained gel; right, autoradiogram; arrow indicates MBP. Note that ERK1 can phosphorylate SEK1(K $\rightarrow$ R) on two consensus MAPK recognition sites at positions 385–391 (right panel, lane 3, and data not shown), although the function of this phosphorylation remains to be determined.

METHODS. 293 cells were transfected as described in Fig. 2 legend. For the Abl experiment, pGNG (SH3-deleted *abl* gene) was transfected (5 μ g). Anisomycin treatment and assays are described in Fig. 2 legend.



in vivo activation of SEK1 by SAPK agonists and the ability of dominant-negative SEK1 to inhibit SAPK activation by extracellular agonists, we propose that SEK1 is a physiological activator of the SAPKs *in vivo*. We chose the name SEK1 (for SAPK/ERK kinase) because other extracellularly regulated kinases (ERKs) have been isolated¹⁷⁻¹⁹ and we do not know yet if these are physiological substrates of SEK1. The inability of Ras and Ras-coupled agonists to activate SEK1 or the SAPKs strongly *in vivo*, coupled with the inability of SEK1(K→R) to block Ras activation of MAPKs and the inability of Raf-1 to activate the SAPKs²⁰, indicate that SEK1 and the SAPKs lie on a signalling pathway that is largely distinct from the Ras/Raf/MEK/MAPK

pathway. In an accompanying paper, we demonstrate that SEK1 is phosphorylated and activated in intact cells not by Raf-1, but by MEK-kinase-1 (ref. 20), a mammalian homologue of the STE11 kinase from *Saccharomyces cerevisiae*²¹. These results support the contention that at least two signal transduction cascades exist in mammals with distinct functions: the MEKK/SEK/SAPK-mediated stress response and the Raf/MEK/MAPK-mediated mitogenic response. The specificity of SEK1 and the MEKs defines the segregation of the stress and mitogenic pathways. Thus, earlier work in yeast, demonstrating multiple independent but homologous signalling pathways (reviewed in ref. 22) can now be extended to mammalian systems. □

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Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1

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A KINASE distinct from the MEK activator Raf¹⁻³, termed MEK kinase-1 (MEKK), was originally identified by virtue of its homology to kinases involved in yeast mating signal cascades⁴. Like Raf, MEKK is capable of activating MEK *in vitro*^{4,5}. High-level expression of MEKK in COS-7 cells⁴ or using vaccinia virus vectors⁵ also activates MEK and MAPK, indicating that MEKK and Raf provide alternative means of activating the MAPK signalling pathway. We have derived NIH3T3 cell sublines that can be induced to express active MEKK. Here we show that induction of MEKK does not result in the activation of MAPK, but instead stimulates the stress-activated protein kinases (SAPKs)⁶⁻⁸ which are identical to a Jun amino-terminal kinase^{9,10}. We find that MEKK regulates a new signalling cascade by phosphorylating an

SAPK activator, SEK1 which in turn phosphorylates and activates SAPK.

Stably transfected NIH3T3 subclones express MEKK in response to isopropyl-β-D-thiogalactoside (IPTG) (Fig. 1a) but MAPK activity remains unchanged (Fig. 1b). In contrast, SAPK activity is increased six- to eightfold in MEKK-inducible cell lines but not in the parent NIH3T3 cells. These MEKK-expressing cells are able to activate MAPK in response to some mitogenic signals, because treatment with phorbol ester increases MAPK activity in each of these clones (as well as in NIH3T3 cells), whereas SAPK activity is unaffected (Fig. 1c). Increased SAPK activity is evident by 3 hours and maximal after 12 hours of induction; MAPK activity is unchanged throughout the 23-hour incubation with inducer (Fig. 1d). Together these results indicate that, in contrast to the presumed role of MEKK in activating MEK and MAPK, MEKK acts instead to activate SAPKs. Expression of truncated ΔMEKK in these clones resulted in six- to eightfold inhibition of growth rate compared with parental NIH3T3 cells.

We modelled activation of SAPK by MEKK using cloned genes and purified proteins expressed using vaccinia virus vectors^{11,12}. MEKK induced electrophoretic retardation of SAPK, which was suggestive of quantitative phosphorylation (Fig. 2a), and also increased the amount of phosphotyrosine in SAPK and activated its Jun N-terminal kinase activity. Thus, in this overexpression model as well as in the inducible cell line, MEKK expression results in activation of the SAPK pathway.

We considered the possibility that activation of SAPK occurred as a consequence of activation of the MEK and MAPK cascade. To stimulate MAPK independently of MEKK, we used activated Raf and a constitutively active allele of MEK1 termed MEK 2E (ref. 5). Both Raf and MEK 2E were able to induce phosphorylation of coexpressed MAPK (Fig. 2b). Neither of these MAPK activators induced phosphorylation of SAPK, indicating that the SAPK activation pathway is effectively insulated from the MAPK pathway.

MEKK was unable to phosphorylate SAPK *in vitro* (below). We therefore tested whether MEKK activated the newly identified SAPK activator, SEK1 (ref. 13), whose sequence is similar to MEK1. Immunopurified MEKK (but not the inactive mutant

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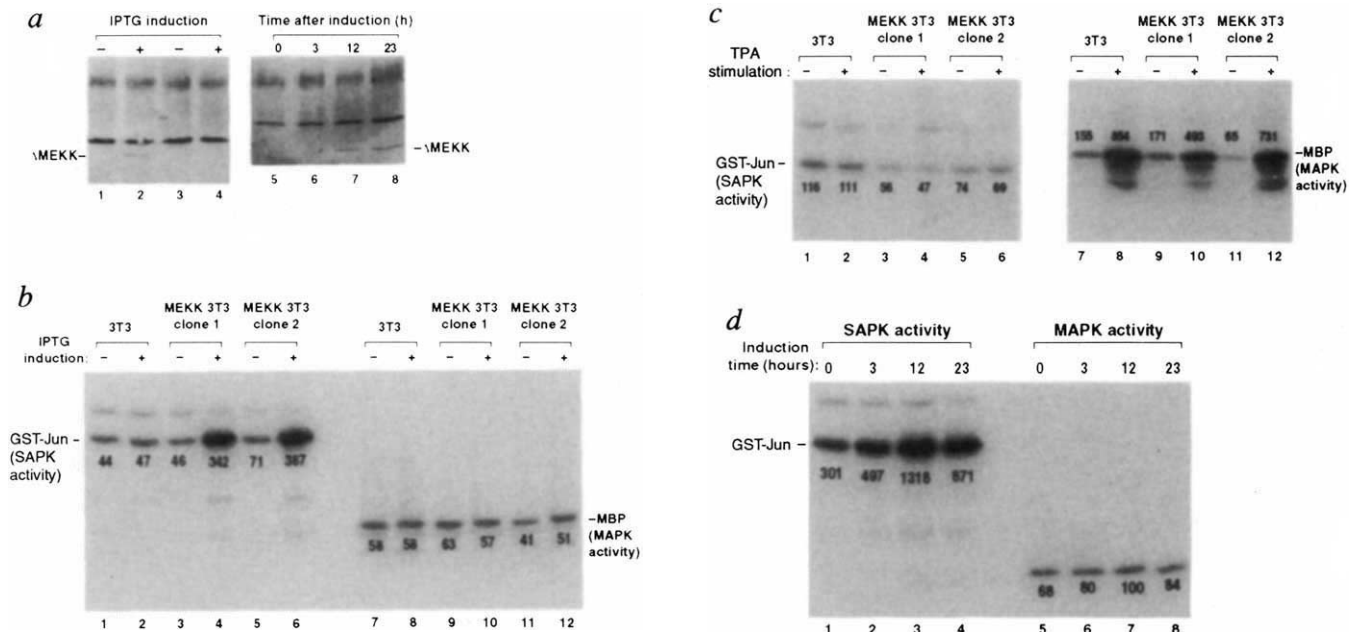


FIG. 1 **a**, MEKK expression in NIH3T3 cells. Epitope-tagged truncated MEKK (Δ MEKK) was detected in MEKK clone 1 cells (lanes 1 and 2) or in MEKK clone 2 cells (lanes 5–8) but not in a control clone (lanes 3 and 4) treated with IPTG for 24 h (lanes 2 and 4) or at the indicated times (lanes 6–8). **b**, MAPK and SAPK activity in MEKK-inducible cell lines. Numbers below labelled bands indicate c.p.m. of radioactivity in substrates. SAPK activity, but not MAPK activity, was increased in response to MEKK expression. **c**, Functional MAPK signalling in NIH3T3 cells and MEKK-expressing subclones after stimulation (+) with 250 ng ml⁻¹ TPA. **d**, Time course of induction of SAPK activity in MEKK 3T3 clone 2 cells after IPTG treatment. MAPK activity throughout this period remained unchanged, whereas SAPK activity was increased even at the 3-h time point, when MEKK expression could not yet be detected.

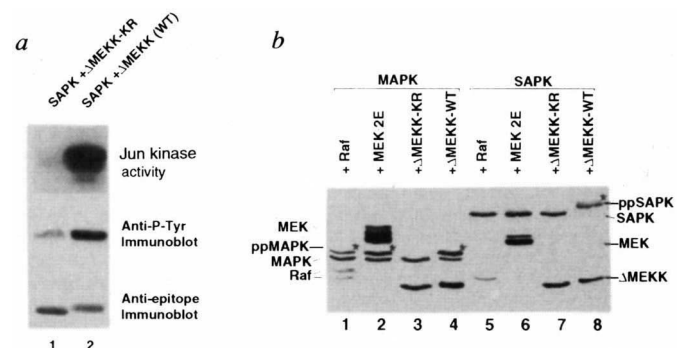
MEKK(K $\rightarrow$ R) rapidly phosphorylated a glutathione-S-transferase (GST)–SEK fusion protein on serine and threonine residues (Fig. 3a) but failed to phosphorylate a SEK mutant in which the two residues equivalent to the sites of activation in MEK were mutated. Phosphorylation of GST–SEK activated the SAPK activity of GST–SEK. Thus, SEK is a substrate of MEKK and phosphorylation by MEKK is sufficient to activate SEK. MEKK expression also activates SEK *in vivo* (Fig. 3b). Activation of SAPK by MEKK requires functional SEK because coexpression of a dominant inhibitory allele of SEK blocks activation of SAPK by MEKK (Fig. 3c).

FIG. 2 Activation of SEK–SAPK pathway by coexpression of Δ MEKK using vaccinia virus vectors. **a**, Epitope-tagged SAPK was expressed with either the untagged inactive K $\rightarrow$ R mutant of Δ MEKK (lane 1) or wild-type Δ MEKK (lane 2). Coexpression of active MEKK resulted in mobility shift of SAPK detected by anti-epitope immunoblotting (bottom panel) and also increased tyrosine phosphorylation of SAPK detected in anti-epitope immunoprecipitates (middle panel). SAPK activity was also strongly elevated, reflected by phosphorylation of GST–Jun(5–89) using anti-epitope immunoprecipitates (top panel). **b**, epitope-tagged MAPK (lanes 1–4) or SAPK (lanes 5–8) was expressed with epitope-tagged forms of truncated active Raf (lanes 1, 5), constitutively active MEK 2E (lanes 2, 6), Δ MEKK(K $\rightarrow$ R) mutant (lanes 3, 7) or Δ MEKK wild type (lanes 4, 8), and detected in whole cell lysates using anti-epitope western blot. Activation of both MAPK and SAPK is identifiable by the appearance of bands with delayed mobility, indicated by stars. pp prefix, phosphorylated protein forms. Raf and active MEK 2E are able to activate MAPK, but not SAPK, thus the SAPK pathway is insulated from the MAPK pathway. MEKK is able to activate MAPK in this overexpression system, though it is not when expressed at lower levels (Fig. 1). Of the kinases tested, only MEKK is able to activate SAPK. METHODS. The N-terminal EE-epitope-tagged p54SAPK α 1, and un-

MEKK expression in cells used here is shown in **a**, lanes 5–8. METHODS. The EE epitope-tagged⁵ C-terminal 320 amino acids of MEKK1 (Δ MEKK) was expressed in NIH3T3 cells using the lacSwitch promoter (Stratagene). Δ MEKK was induced in cell clones with 1 mM IPTG and detected by immunoprecipitation and immunoblotting using the anti-EE monoclonal antibody (mAb). MAPK and SAPK activity was determined using polyclonal antibodies recognizing a C-terminal peptide of p42MAPK or a p54SAPK–GST fusion protein. Immune complexes containing protein from 10⁵ cells were reacted with 0.5 μ g GST–Jun (amino acids 5–89; ref. 8) for SAPK, or MBP (Sigma) for MAPK in 20- μ l reactions (50 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, 1 mM DTT, 15 μ M ATP, 5 μ Ci [³²P- γ]ATP), for 30 min at room temperature. Radioactivity was quantified using an AMBIS β -detector.

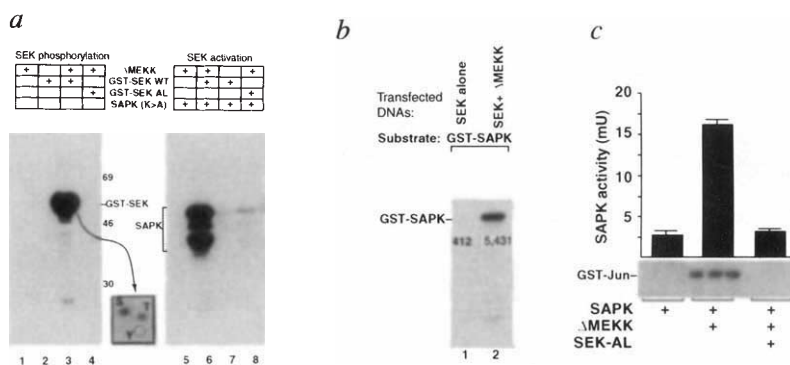
Coexpression of full-length MEKK protein is able to effect phosphorylation of SEK and activation of SAPK (Fig. 4a), similar to the activation induced by truncated MEKK. The high activity of full-length MEKK protein during overexpression suggests that a cellular activity might regulate natural MEKK expressed at lower levels. This result is in contrast to Raf, which displays low levels of kinase activity unless truncated¹⁴.

Our results demonstrate complete reconstitution of a kinase cascade, beginning with MEKK, that phosphorylates and activates SEK, which subsequently phosphorylates and activates SAPK. Each component of this cascade is functionally parallel



tagged Δ MEKK were expressed using the vaccinia virus expression system and the plasmid pTM1 (ref. 12). Phosphotyrosine was detected using mAb 4G10 (UBI) to probe anti-EE immunoprecipitates. Jun kinase was assayed as for Fig. 1. Kinase expression and blotting has been described⁵.

FIG. 3 a, MEKK phosphorylates and activates of SEK *in vitro*. Immunopurified Δ MEKK phosphorylated wild-type GST-SEK (lane 3) on serine and threonine (see phosphoaminoacid analysis, inset), but not mutant GST-SEK protein lacking the two phosphorylation sites (lane 4). GST-SEK1 phosphorylated by MEKK *in vitro* acquired SAPK kinase activity, as shown after secondary reaction with inactive (K>A) mutant thrombin-cleaved GST-SAPK with radioactive ATP (lane 6). Mutant GST-SEK protein lacking phosphorylation sites (lane 8) or reactions without either MEKK or SEK did not allow phosphorylation of SAPK. b, MEKK activates SEK1 *in vivo*. Epitope-tagged SEK expressed in CV1 cells using a CMV expression vector (lane 1) became activated by coexpression of Δ MEKK lacking the epitope tag (lane 2). Anti-epitope immunocomplexes were assayed for SEK activity using GST-SAPK as substrate (see Fig. 1 legend). c, SAPK activation by MEKK requires SEK1. Epitope-tagged SAPK expressed using SV40-based vectors was activated by coexpression with Δ MEKK. This activation was reversed by triple coexpression of a dominant inhibitory mutant of SEK1 containing (S220A, T224L; SEK AL).



to a component of the MAPK activation pathway: SAPK is analogous to MAPK, SEK analogous to MEK, and MEKK analogous to Raf (Fig. 4b). Other evidence suggests that SAPK signalling in response to ultraviolet irradiation^{10,15} and tumour-necrosis factor- α ¹⁶ lies downstream of Ras. Additionally, dominant inhibitory Ras reduces the activity of MEKK¹⁷ and the MEKK homologue Byr2 associates with Ras1 in yeast¹⁸. Thus, in parallel to Raf-MEK-MAPK, the MEKK-SEK-SAPK pathway most probably lies downstream of Ras. Cofactors for Ras must exist that contribute specifically to either the mitogenic or stress-response pathways.

concentration). Inhibition of HA-epitope-tagged SAPK by SEK-S220A, T224L (SEK-AL) was tested in L929 cells using the SV40-based pMT2 vector; 7 μ g SAPK, 7 μ g Δ MEKK and 15 μ g SEK-AL expression plasmids were transfected together with empty pMT2 vector DNA to equalize plasmid mass. SAPK assays represent triplicate measurements using GST-Jun substrate as described¹³.

Is MEKK able to activate MEK, as originally proposed? When tested *in vitro* or during overexpression^{4,5}, MEKK is able to phosphorylate and activate MEK. But with the stable MEKK-inducible NIH3T3 cells studied here, MEKK, through SEK, activates SAPK and not MAPK, even though these cells express an intact MAPK activation pathway. MEKK might stimulate MAPK in other cell types, or transiently, although we detected no MAPK activity as early as 3 hours, at which time SAPK activity was raised and MEKK was undetectable. Additionally, activated *Drosophila* MEK (Dsr1) rescues D-Raf null mutants in both the Torso and R7 photoreceptor pathways¹⁹, implicating Raf as the major physiological MEK activator.

Perhaps it is appropriate to consider MEKK versus RAF signalling as analogue rather than binary cell regulation. Depending on the interplay between kinase activities, substrate availability and the intracellular milieu, MEKK might activate SAPK in our experimental systems; other cellular conditions might translate MEKK activity into a variety of mixed signals involving other homologues of MAPK²⁰, including SAPK. □

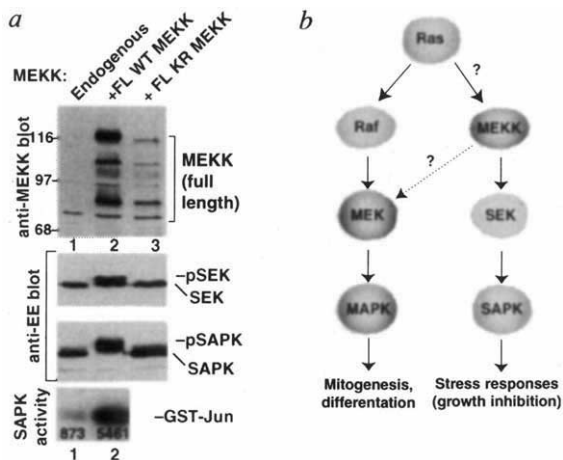


FIG. 4 a, Activation of SEK and SAPK *in vivo* by full-length MEKK. Using the vaccinia virus expression system in CV1 cells, SEK1 or SAPK were separately expressed alone (lane 1) or with vectors encoding full-length wild-type MEKK1 (lane 2) or a kinase-inactive (K447R) mutant allele of MEKK1 (lane 3). Expression of several bands related to full-length MEKK (top panel) was detected using chicken polyclonal antibodies raised against bacterially expressed Δ MEKK. Endogenous proteins of 70K, 90K and 110K were also detected (lane 1). Epitope-tagged SEK1 and SAPK1 (middle panels) were detected by immunoblotting. Electrophoretically retarded bands arising from phosphorylation of both SEK and SAPK were observed from coexpression with full-length MEKK, indicating that MEKK thus expressed is constitutively active. SAPK activity was activated by full-length MEKK (bottom panel). b, Diagram of separate pathways emanating from Raf and MEKK. Both Raf and MEKK appear to be dependent upon the function of Ras, as described in the text. The Raf-MEK-MAPK pathway is functionally analogous to the MEKK-SEK-SAPK pathway, although the result of stimulation of each pathway is distinct. A dotted arrow from MEKK to MEK reflects the ability of MEKK to phosphorylate MEK *in vitro* and during high-level cell expression. In stable inducible cell lines, MAPK activation by MEKK is not seen, drawing the physiological significance of this path into question. The opposing nature of two signalling pathways both emanating from Ras suggests an important role for factors cooperating with Ras to provide specificity for stimulation of one path versus the other.

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Involvement of microtubule motors in basolateral and apical transport in kidney cells

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THE maintenance of a polarized cell surface requires vectorial transport of vesicles to the apical and the basolateral membrane domains¹. Transport of newly synthesized apical proteins and trans-cytosis from the basolateral to the apical surface have been demonstrated to depend on microtubules²⁻⁹. In contrast, movement of membrane proteins to the basolateral surface has been claimed to occur by diffusion and to be microtubule- and actin-independent^{2,4,5,7,10-12}. We have re-examined the role of microtubules using a recently developed polarized transport assay in permeabilized Madin-Darby canine kidney cells^{13,14}. Here we report that both apical and basolateral transport is inhibited by nocodazole treatment. Transport to the basolateral surface was inhibited by immunodepletion of cytosolic kinesin. In contrast, apical transport involved both dynein and kinesin. Our data demonstrate that in epithelial cells, microtubule motors are involved in the movement of apical and basolateral vesicles. Moreover, we propose that the differential requirement for microtubule-based motors is related to the microtubule organization.

We studied the influence of microtubules in vectorial vesicular traffic, taking advantage of a polarized transport assay dependent on temperature, energy and exogenously supplied cytosol^{13,14}. For the assay we used fully polarized Madin-Darby canine kidney (MDCK) cells. Transport from the *trans*-Golgi network (TGN) to the different membrane domains was determined by following appearances of vesicular stomatitis virus glycoprotein (VSV-G) to the basolateral surface and influenza virus haemagglutinin (HA) to the apical surface. Cells were infected, pulse-labelled, chased at 20 °C and, on either the apical or the basolateral side, permeabilized by chromatographically purified streptolysin O (SLO). The endogenous cytosol was washed out and transport was reconstituted at 37 °C in the presence of exogenously added cytosol supplemented with an ATP-regenerating system. Basolateral transport was measured by surface immunoprecipitation of VSV-G whereas apical transport was quantified by the trypsin sensitivity of HA. Transport is defined as the increase in transported product due to the addition of cytosol¹⁴. Usually, upon cytosol addition, the apical transport is increased twofold (1.7 ± 0.3 , $n = 30$) and the basolateral transport 2.5-fold (2.2 ± 0.6 , $n = 20$) (see also refs 13, 14).

This *in vitro* transport assay offers the possibility to quantify how various reagents or experimental manipulations affect the ability of cytosol to stimulate transport. We use nocodazole (NZ) to test whether microtubules play a role in polarized transport. NZ completely inhibited apical transport of HA (Fig. 1a) as previously reported^{2,7}. Surprisingly, NZ inhibited the basolateral transport of VSV-G (Fig. 1b). This result is at variance with previously published results in epithelial cells^{2,4,5,7,10-12}. As it is difficult completely to depolymerize microtubule with drugs^{15,16}, it is possible that in previous studies, microtubule depolymerization was incomplete. This possibility was never rigorously excluded^{3,5,17}. Moreover, our results are in agreement with the *in vitro* binding of basolateral exocytic vesicles to microtubules¹⁶. Therefore, we checked the integrity of microtubules in intact cells and cells permeabilized and processed as for the transport assay (see details in Fig. 2). After transport, cells were fixed

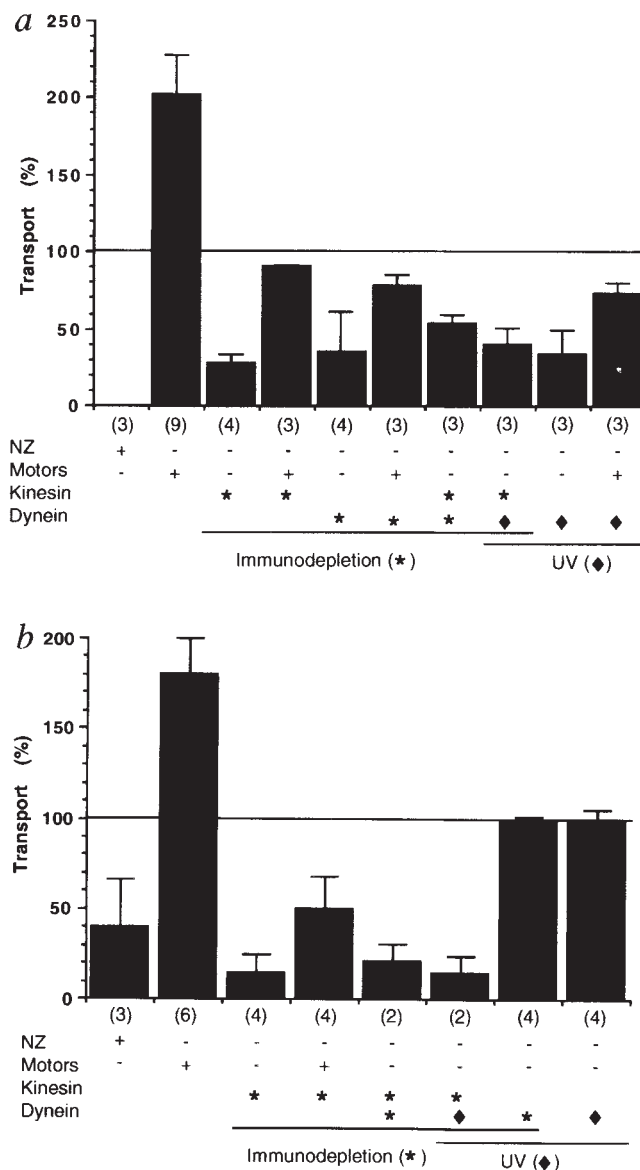


FIG. 1 a, *In vitro* transport of HA from the TGN to the apical surface is inhibited by nocodazole (NZ), and partially inhibited by kinesin or dynein immunodepletion and by dynein photobleaching. Inhibition due to kinesin or dynein depletion could be partially rescued by addition of a motor-enriched preparation. b, *In vitro* transport of VSV-G from the TGN to the basolateral surface is partially inhibited by nocodazole (NZ) or kinesin immunodepletion. This latter inhibition could be partially rescued by addition of a motor-enriched preparation.

METHODS. *In vitro* transport of VSV-G or HA was done on permeabilized and cytosol-depleted MDCKII cells grown on filters, with the following modifications to the procedures described elsewhere^{13,14}. Nocodazole (33 μ M from a stock solution in DMSO) was present during the last 90 min of the viral replication at 37 °C; the 60 min chase at 20 °C; 30 min on ice (an additional step introduced in the assay); the depletion of endogenous cytosol (30 min at 20 °C) and the 45–50 min transport at 37 °C. All samples were analysed by 10% SDS-PAGE, scanned with a PhosphorImager and quantified using the ImageQuant software (Molecular Dynamics). The amount of VSV-G or HA transported was calculated as VSV-G % transport = (VSV-G surface immunoprecipitated/VSV-G total) $\times$ 100 and HA % transport = $(2 \times \text{HA2}/\text{HA} + 2\text{HA2}) \times 100$ where HA2 is the trypsin cleavage product of HA¹³. The values are expressed as control cytosol-dependent transport being 100% (transport in the presence of control cytosol minus transport in the absence of added cytosol). For each manipulation, a matched control was used. Values are means $\pm$ s.e.m. for experiments done with duplicate filters. Number of experiments performed per condition is indicated in parentheses. HeLa cytosol was obtained following a protocol described elsewhere¹⁴. The crude motor-enriched preparation was obtained from rat liver using a described protocol³⁰. Photocleavage of cytosolic dynein heavy chains was according to published procedures^{22,23}. HeLa cytosol was depleted of kinesin using the SUK4 monoclonal IgG coupled to Sepharose beads following published protocols^{19,20}. The same procedure was followed to deplete cytosolic dynein with the 70.1 monoclonal IgM (Sigma) coupled to goat anti-mouse agarose beads (Sigma)²⁰. Monoclonal IgG p5D4 and IgM 5D3 were used as a control²⁰.

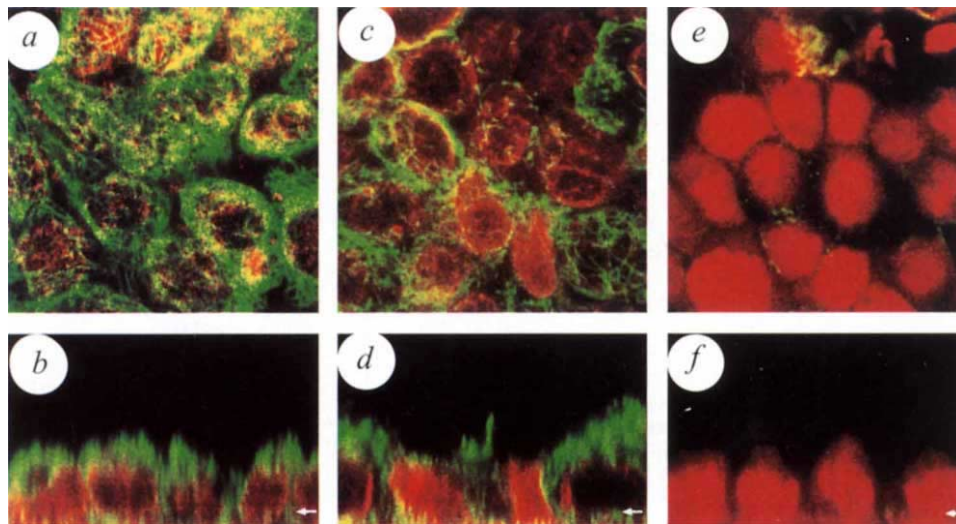


FIG. 2 α -Tubulin (green) and propidium iodide (red) double staining of VSV-infected MDCKII cells: intact (a, b), apically SLO permeabilized (c, d) or both apically permeabilized and nocodazole treated (e, f). Images presented are confocal x, y sections (a, c, e) taken at the level of the arrows indicated in the corresponding z sections (b, d, f). METHODS. MDCKII cells grown on filters were processed exactly as for transport assays except that radioactive metabolic labelling, immunoprecipitation and cell lysis were omitted. Cells were either permeabilized on their apical surface or left intact and those permeabilized either

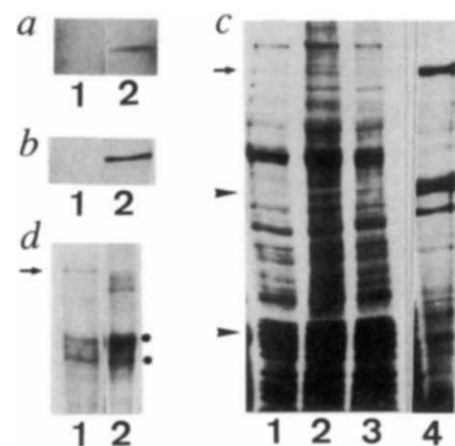
treated with nocodazole or not. After the transport reaction, cells were fixed using the pH-shift paraformaldehyde method and processed for double staining as described elsewhere¹⁸. Microtubules were stained with monoclonal anti- α -tubulin antibody (Amersham) and with anti-mouse Ig-FITC conjugated antibody (Dianova). DNA was stained using propidium iodide. Confocal laser scan microscopy was with the EMBL compact confocal system. Photomultiplier gain and laser power were identical in all observations. Images were photographed with a Polaroid freeze-frame directly from the monitor.

following the pH-shift paraformaldehyde method and double stained for microtubules and DNA¹⁸. Staining observed with VSV-infected cells is presented here but similar results were obtained after infection with influenza virus (not shown). Compared to intact cells (Fig. 2a, b), SLO permeabilization (Fig. 2c, d) led to partial microtubule disassembly. Although NZ treatment of intact cells did not result in complete disappearance of microtubules (not shown, ref. 15), after both NZ treatment and SLO permeabilization almost no microtubule staining was observed (Fig. 2e, f). We think that prolonged NZ treatment combined with permeabilization more effectively depolymerized

microtubules explaining the observed impairment of basolateral transport of VSV-G.

Because NZ inhibited transport in the permeabilized cell system, we sought to determine whether microtubule motors function in vesicular traffic. Toward this end, we depleted cytosol of kinesin or dynein and used these depleted cytosols in the polarized transport assay. HeLa cytosol was immunodepleted of kinesin using the monoclonal IgG antibody SUK4 (Fig. 3a)^{19,20}. To deplete cytosol of dynein two methods were applied. First, dynein intermediate chain was immunodepleted with the monoclonal IgM antibody 70.1 (Fig. 3b)^{20,21}. Second, ultraviolet

FIG. 3 a, Kinesin heavy-chain immunodepletion from HeLa cytosol with the monoclonal IgG SUK4 (lane 1) or HeLa cytosol incubated with the irrelevant IgG p5D4 (lane 2) as analysed by western blotting with the SUK4 antibody. Bands were detected using anti-mouse horseradish peroxidase conjugated antibodies (Dianova) and ECL (Amersham). b, Cytoplasmic dynein intermediate-chain immunodepletion from HeLa cytosol with the monoclonal IgM 70.1 (lane 1) or HeLa cytosol incubated with the irrelevant IgM 5D3 (lane 2) as analysed by western blotting with the 70.1 antibody. c, Ultraviolet mediated photocleavage of cytoplasmic dynein heavy chains. HeLa cytosol (lane 1) was treated with UV in the presence of 100 μ M vanadate, 1 mM ATP and 5 mM noradrenaline (NA, lane 2) or NA was added after the UV-mediated irradiation (lane 3). Samples were electrophoresed on 4% acrylamide gel and the gel silver stained. One band at the mobility of cytoplasmic dynein heavy chains (arrow, M_r = 360K) was significantly reduced (lane 3) when compared with control cytosol (lane 1) and presumably reflected the photocleavage of cytoplasmic dynein. This photocleavage is prevented when cytosol is treated with UV light in the presence of NA (lane 2). The abundance of the protein was too low to be detected unambiguously by the appearance of the lower M_r cleavage products in a region of the gel containing many other polypeptides. Molecular size standards are indicated by arrowheads and correspond to M_r = 205K and M_r = 116.5K. Lane 4 shows a preparation enriched in motors. d, Immunoprecipitation of NA-protected and photocleaved cytosols with the monoclonal IgM 70.1 antibody. Samples were electrophoresed in a 4% acrylamide gel and



the gel silver stained. Lane 1 shows the cytoplasmic dynein heavy chain (arrow) in the NA-protected UV-irradiated sample which is almost undetectable after photocleavage (lane 2) even if the photocleaved sample is overloaded as determined by the amount of dynein intermediate chain immunoprecipitated (*). Only relevant parts of gels and immunoblots are shown.

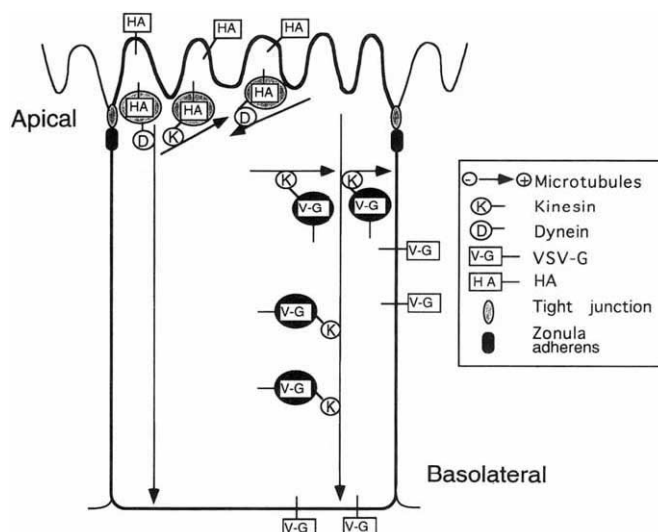


FIG. 4 Working model for microtubule motor dependence of VSV-G basolateral and HA apical transport. The microtubule 'plus-end directed' kinesin is proposed to move vesicles carrying VSV-G to the basolateral plasma membrane along uniformly apical to basal microtubule bundles. Both kinesin and the microtubule 'minus-end directed' dynein would be needed for apical vesicles carrying HA to cross the dense mat of microtubules located between the Golgi apparatus and the apical plasma membrane.

irradiation in the presence of vanadate specifically cleaved cytosolic dynein heavy chain (Fig. 3c,d), inactivating its motor activity^{21,22}. As a control, dynein was protected from the photocleavage by the addition of noradrenaline (NA) (Fig. 3c,d)^{22,23}. NA reduces vanadate to oxyvanadate which neither mediates UV-photocleavage nor acts as an enzyme inhibitor²². We measured delivery of VSV-G to basolateral surfaces after kinesin heavy-chain immunodepletion and found a significant inhibition of transport (Fig. 1b). Moreover, addition of a preparation enriched in motors (50 $\mu\text{g ml}^{-1}$ of both kinesin and dynein) increased the transport and partially rescued the inhibition due to kinesin depletion (Figs 1b and 3c). In contrast, neither dynein immunodepletion nor photocleavage modified the basolateral transport of VSV-G (Fig. 1b). When apical transport of HA was assayed, we found a significant inhibition with both kinesin- or dynein-immunodepleted cytosols (Fig. 1a). Consistent with this result, inhibition of HA transport was also found after dynein photocleavage (Fig. 1a). The impaired apical transport due to kinesin or dynein depletion could be partially rescued by the motor preparation (Fig. 1a). This preparation increased the apical transport when added to normal cytosol (Fig. 1a).

The most likely interpretation for the differential requirement of kinesin and dynein in polarized surface transport resides in the organization of the microtubule network (Fig. 4). In MDCK cells, microtubules are organized in longitudinal bundles with most of the plus-ends of the microtubules directed towards the basal surface¹⁵. There is also a dense meshwork of microtubules of opposite polarity above the nucleus²⁴. We propose that basolateral vesicles use kinesin-driven transport to move both along longitudinal microtubule bundles to gain access to the basal surface and along transverse microtubules above the nucleus to reach areas close to the junctional complexes. Because basolateral vesicles use kinesin, could they be mislocalized to the apical membrane by moving along those microtubules that have their plus-ends apically? Previous reports have demonstrated that missorting of basolateral proteins is very low¹. Mistargeting of the vesicles could be hindered by the presence of the subapical network of actin filaments, the crossing of which may require specific myosin motors^{17,25}.

Surprisingly we found that transport of apical vesicles depends to the same extent on either kinesin or dynein. Because depletion of both kinesin and dynein did not fully inhibit apical transport (Fig. 1), this would argue for the presence of both motors on the same vesicle (compare with ref. 26). However, more evidence will be needed to demonstrate that both motors are active on the same vesicle. Because apical vesicles seem to use both motors, how can mislocalization of these vesicles to the basolateral membrane be prevented? There is often a small fraction of apical proteins being delivered to the 'wrong' membrane¹. Whether this

is due to protein missorting in the TGN or to mistargeting of apical transport vesicles is not known. It is also possible that an apical vesicle that has been translocated erroneously by its kinesin motor to the vicinity of the basolateral membrane will not be able to dock to the membrane because it does not contain the appropriate docking proteins, such as basolateral v-SNARE²⁷. An alternative mechanism to achieve correct delivery of vesicles to the apical side may reside in the organization of microtubules in different regions of the cell. Apical vesicles may not be able to bind the longitudinal microtubule bundles which may have a different microtubule-associated protein (MAP) composition than the microtubules of the apical meshwork. Note that in neurons, axonal and dendritic microtubules are decorated with different MAPs²⁸. Recently, a MAP specific for a subset of microtubules in epithelial cells was identified²⁹. Further work will be necessary to find out whether movement along microtubules is regulated by specific MAPs. □

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XLas is a new type of G protein

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THE GTP-binding proteins are well-known regulators of cellular functions¹⁻⁴, including vesicular transport⁵⁻⁷. Cholera toxin, which is known to catalyse ADP-ribosylation of the α s subunit of heterotrimeric G proteins, stimulates secretory vesicle formation from the *trans*-Golgi network⁸. Here we describe a new cholera toxin target, an 'extra large' G protein (XLas; M_r 92K) which consists of a new 51K XL-portion linked to a Gas truncated at the amino terminus. XLas is specifically associated with the *trans*-Golgi network and occurs selectively in cells containing both the regulated and the constitutive pathway of protein secretion. Hence, XLas may mediate the effects of cholera toxin on secretory vesicle formation.

To search for the possible presence of proteins that, in addition to α s, undergo ADP-ribosylation by cholera toxin in PC12 cells *in vivo*, we incubated cells in the absence or presence of cholera toxin and then subjected a postnuclear

supernatant (PNS) prepared from these cells to 'back-ribosylation' *in vitro* using cholera toxin and [³²P]NAD. With this approach, a protein that has become ADP-ribosylated by cholera toxin *in vivo* will show a reduced incorporation of [³²P]ADP-ribose in the subsequent *in vitro* reaction. Analysis of a membrane pellet derived from the PNS showed that PC12 cells contain a protein with an apparent M_r of 94K (referred to as XLas, for 'extra large' α s) that, in addition to the α s doublet, is a major *in vivo* target for ADP-ribosylation by cholera toxin (Fig. 1a).

Peptide mapping suggested that XLas contains certain domains that are homologous to α s and others that are not (data not shown). XLas was immunoprecipitated with antibodies against the carboxy-terminal decapeptide of α s and against an epitope of α s that is encoded by exon 2 of the α s gene (Fig. 1b). Immunoprecipitation was prevented by the addition of the peptides used to raise the respective antibodies. In contrast to α s, however, XLas was not immunoprecipitated with an antibody against an epitope of α s that is encoded by exon 1 of the α s gene. These results suggested that XLas is highly homologous, if not identical, to α s with respect to exon 2, exon 8 (ADP-ribosylation site) and exon 13 (C terminus), but contains new sequence instead of that encoded by exon 1.

In northern blot analysis of PC12 cell RNA, oligonucleotide probes for either exon 1 or exon 2 of α s (Fig. 2a) both detected

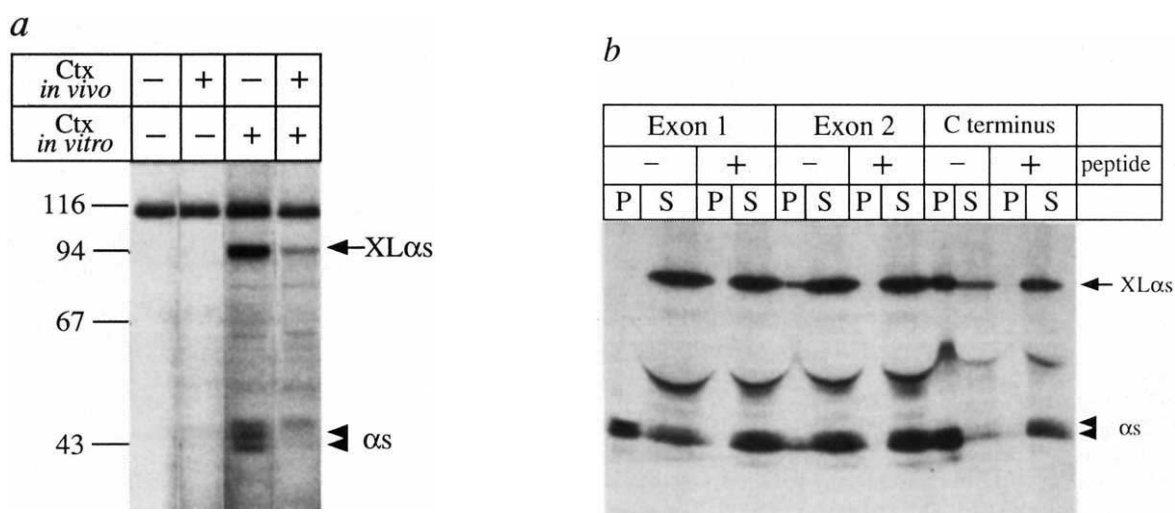


FIG. 1 XLas is a new cholera toxin target related to α s. **a**, *In vitro* ADP-ribosylation of membrane proteins with [³²P]NAD in the absence (–) or presence (+) of cholera toxin (Ctx) following *in vivo* treatment of PC12 cells without (–) or with (+) cholera toxin. The [³²P]ADP-ribosylated 115K band is probably auto-ADP-ribosylated poly(ADP-ribose) polymerase²³ derived from broken nuclei. **b**, Immunoprecipitation of [³²P]ADP-ribosylated membrane proteins of PC12 cells using antibodies raised against peptides corresponding to a portion of exon 1 of α s (Glu 27–Arg 42, see Fig. 2e), of exon 2 (Gly 47–Met 60, corresponding to Gly 499–Met 512 of XLas, see Fig. 2c) and to the C-terminal decapeptide of α s (which is identical to that of XLas, see Fig. 2c). Immunoprecipitation was done in the absence (–) or presence (+) of the respective peptides. P, Immunoprecipitate; S, supernatant after immunoprecipitation.

METHODS. **a**, PC12 cells were treated *in vivo* with or without cholera toxin overnight, and a PNS was prepared as described^{8,24}. For *in vitro* [³²P]ADP-ribosylation, 90 μ l PNS (~2 mg protein per ml in 0.25 M sucrose, 10 mM HEPES–KOH, pH 7.4, 1 mM magnesium acetate (MgOAc₂), 1 mM EDTA, 10 mM thymidine, 1.6 mM Na₂HPO₄, 0.5 mM PMSF) were incubated in the absence or presence of activated cholera toxin (5 μ g ml^{–1}) in a final volume of 100 μ l containing in addition an ATP-regenerating system²⁴, a final free Mg²⁺-concentration of 200 μ M, 250 μ M dithiothreitol (DTT) (from the activation of cholera toxin), 10 μ M unlabelled NAD and 50 μ Ci ml^{–1} [³²P]NAD (800 Ci mmol^{–1}). After 1 h at 37 °C, membranes were pelleted (10 min, 14,000g, 4 °C) and analysed

by SDS–PAGE followed by autoradiography. **b**, Membranes (300 μ g protein) prepared from a PC12 cell PNS (10 min, 14,000g, 4 °C) were incubated in a final volume of 600 μ l containing 300 mM sodium phosphate, pH 7.2, 25 mM Tris–HCl, pH 7.4, 10 mM MgCl₂, 1 mM EDTA, 10 mM thymidine, activated cholera toxin (5 μ g ml^{–1}), 250 μ M DTT (from the activation of cholera toxin), 1 mM ATP, 0.1 mM GTP, 10 μ M unlabelled NAD and 50 μ Ci ml^{–1} [³²P]NAD (800 Ci mmol^{–1}). After 1 h at 37 °C, membranes were pelleted (10 min, 14,000g, 4 °C), washed once with 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and resuspended at 1 mg protein per ml in the same buffer. Proteins were solubilized by adding four vols immunoprecipitation buffer (100 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1% Triton X-100, 1% sodium deoxycholate, 0.5% SDS and 1 mM PMSF). Aliquots of the solubilized proteins were incubated overnight with the rabbit antibodies (3–6 μ l serum per 350 μ l aliquot) in the absence or presence of the peptides (15 μ g ml^{–1}) against which the antibodies were raised. The exon 1 antibody was obtained from T. Pfeuffer and S. Mollner, Düsseldorf; the exon 2 antibody is the one reported in ref. 25 (a gift of G. Schultz), and the antibody against the C-terminal decapeptide of α s was raised as described previously²⁶. Immune complexes (P) were collected using Protein A-Sepharose in immunoprecipitation buffer, whereas the proteins in the supernatant (S) obtained after pelleting the Protein A-Sepharose were precipitated with trichloroacetic acid (TCA), followed in both cases by SDS–PAGE and autoradiography.

the 1.8 kilobase (kb) *as* messenger RNA. However, only the exon 2 probe detected a 3 kb mRNA, that is, an mRNA long enough to code for XLas. This information was used to isolate an XLas complementary DNA. The authenticity of the cDNA was verified by northern blot analysis (Fig. 2a) and immunoprecipitation (Fig. 2b). The XLas cDNA (2,956 nucleotides without the poly(A) tail) contains an open reading frame of 2,538 nucleotides, encoding an 846 amino-acid residue, 92K protein (Fig. 2c). The XLas protein consists of two portions, and N-terminal one containing 498 amino-acid residues, referred to as the XL portion, and a C-terminal one containing 348 residues, referred to as the *as* portion. The XL portion is a new sequence that, as a whole, does not exhibit any significant homology to any of the protein sequences in the databases. It appears to contain several structurally distinct domains (Fig. 2d, see legend for details), an acidic polar N-terminal domain followed by a very alanine-rich domain with EPAA and AARA repeats, a proline-rich hinge, a cysteine-rich domain and finally a domain with homology to exon 1 of *as* (Fig. 2e, see below). The *as* portion of XLas is identical to amino-acid residues 47–394 of *as*⁹, which are encoded by exons 2–13 of the *as* gene. Hence, it contains all the functional domains intrinsic to this principal part of *as*, including those mediating receptor and effector interaction, GTP binding and hydrolysis (see Fig. 2d)¹⁰. Indeed, photoaffinity labelling with [α -³²P]GTP azidoanilide showed that XLas binds GTP (R.H.K., S. Offermanns, G. Schultz and W.B.H., unpublished observations).

The nucleotide sequence encoding the XL portion, towards its 3' end (the junction to exon 2), becomes increasingly homolo-

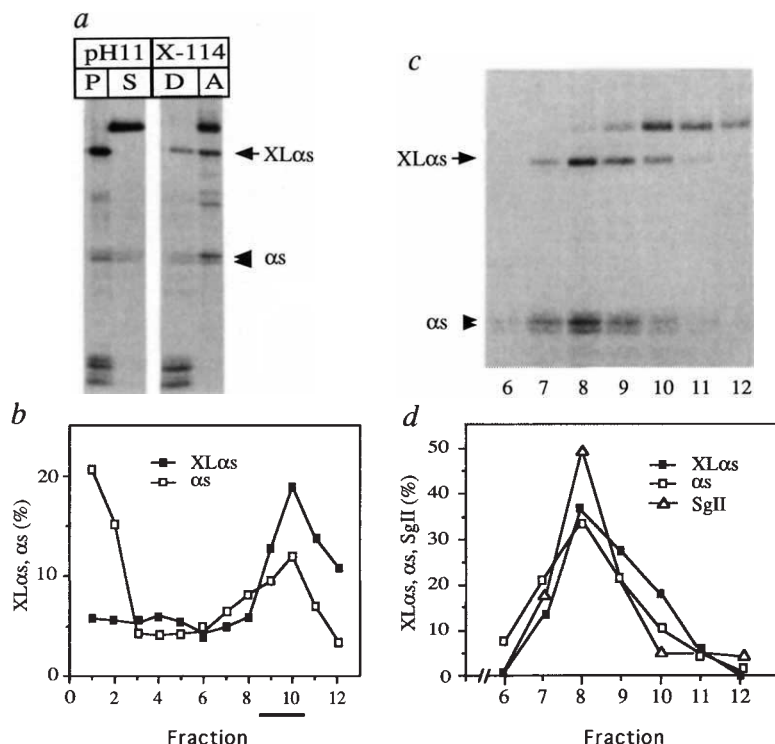
gous to that of exon 1 of *as*. Comparison of the predicted amino-acid sequence of this region with the N-terminal sequences of *as* and other *Ga* subunits (Fig. 2e) reveals that most of those residues that are conserved between the various *Ga* subunits are also conserved in XLas. We think it is likely that this conservation reflects a common function of this domain: the binding of $\beta\gamma$ subunits¹⁰.

As the C-terminal half of XLas is identical in sequence to that encoded by exons 2–13 of the single copy *as* gene^{9,11}, XLas presumably is a product of this gene. Given the lack of exon 1, transcription of the XLas mRNA is likely to start at a site which is different to that giving rise to the *as* mRNA and which is located either 5' of exon 1, with exon 1 being excised during splicing, or within the large intron 1. Evidence for alternative transcriptional start sites in the *as* gene exists. Complementary DNAs derived from the *as* gene have been isolated that predict proteins slightly smaller than *as*^{12,13}. These cDNAs contain unique exons instead of exon 1.

XLas is tightly membrane associated because it was not solubilized by carbonate stripping of membranes at pH 11 (Fig. 3a, left), a condition which solubilized about one third of *as* and which is known to remove most peripheral membrane proteins. When membranes were solubilized in Triton X-114 and the solubilized proteins subjected to phase separation, a significant proportion of XLas was found in the final detergent phase, although a greater proportion was recovered in the final aqueous phase, as was the case for *as* (Fig. 3a, right). Thus, the association of XLas with the membrane seems to involve a hydrophobic interaction which, however, appears to be confined to the cyto-

FIG. 3 Membrane association and subcellular localization of XLas in PC12 cells. a, Carbonate extraction (pH 11) and Triton X-114 phase separation (X-114) of membranes containing [³²P]ADP-ribosylated XLas and *as*. P, Carbonate-insoluble pellet; S, carbonate supernatant; D, Triton X-114 detergent phase; A, Triton X-114 aqueous phase. Note that the [³²P]ADP-ribosylated 115K band is extracted by carbonate and is recovered in the Triton X-114 aqueous phase, whereas the [³²P]ADP-ribosylated bands at the bottom of the gel are not extracted by carbonate and are recovered in the detergent phase. b–d, Subcellular localization of [³²P]ADP-ribosylated XLas as revealed by sequential velocity (b) and equilibrium (c, d) sucrose gradient centrifugation of a PNS. The bar in b indicates the fractions (9 + 10) of the velocity gradient that were loaded onto the equilibrium gradient. c, The autoradiogram of the equilibrium gradient used to obtain the XLas and *as* data plotted in d. The distribution of ³²P-labelled XLas and *as* across the equilibrium gradient (d) is shown in comparison with that of the TGN, identified by [³⁵S]sulphate pulse-labelled secretogranin II (SgII)¹⁴. For each labelled protein analysed, the data shown in b and d are expressed as per cent of total per gradient. Fraction 1 is the top of the gradient.

METHODS. a, Membrane pellets were obtained after cholera toxin-catalysed *in vitro* [³²P]ADP-ribosylation as described in the legend to Fig. 1. For carbonate extraction, membranes (100 μ g protein) were resuspended in 50 μ l H₂O, mixed with 50 μ l of extraction buffer (0.2 M Na₂CO₃, pH 11, 4 mM EDTA, 0.05% saponin, 0.5 mM PMSF), vortexed for 30 min at 4 °C, and centrifuged for 30 min at 100,000g. Pellet (P) and supernatant (S) were analysed by SDS-PAGE followed by autoradiography. For Triton X-114 phase separation, the method of ref. 32 was used with the following minor modifications. (1) 1% Triton X-114 in buffer A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) was used to solubilize the membranes (100 μ g protein). (2) The sucrose cushion was omitted. (3) The first aqueous phase was supplemented with fresh Triton X-114 and the first detergent phase mixed with fresh buffer A. Both samples were then subjected to a second condensation in separate tubes, yielding the second aqueous phase (A) and the second detergent phase (D), respectively. The latter were analysed by SDS-PAGE followed by autoradiography. b–d, A PC12 cell PNS (1.3 ml) was subjected to cholera toxin-catalysed *in vitro*



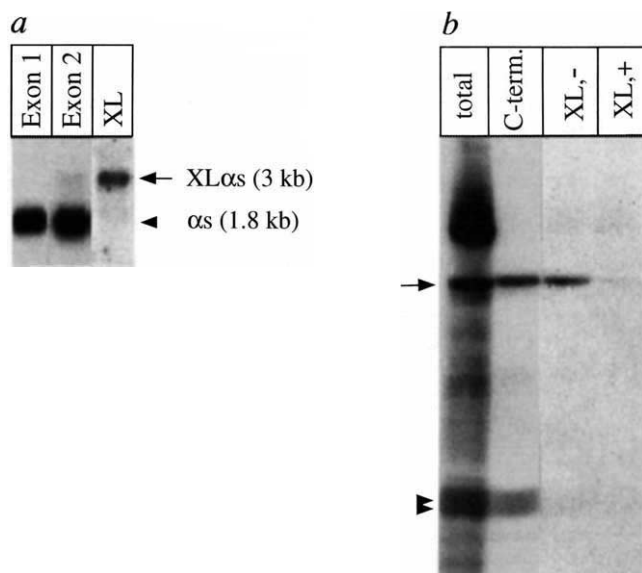
[³²P]ADP-ribosylation as described in the legend to Fig. 1. Alternatively, a PNS was prepared from PC12 cells pulse-labelled for 5 min with [³⁵S]sulphate as described²⁴. Sequential velocity and equilibrium (0.5–1.6 M sucrose) sucrose gradient centrifugation of the two PNSs was done as described²⁴, loading aliquots of fractions 9 and 10 of the velocity gradient onto the equilibrium gradient. Fractions were subjected to SDS-PAGE followed by autoradiography or fluorography, and ³²P-labelled XLas, ³²P-labelled *as* and ³⁵S-labelled secretogranin II (SgII) were quantified by densitometric scanning.

plasmic leaflet of the membrane because XLas does not contain any obvious transmembrane domain.

To determine the subcellular membrane compartment with which XLas is associated, we subjected the PNS of PC12 cells to an established subcellular fractionation protocol which consists of sequential velocity and equilibrium sucrose gradient centrifugation¹⁴. The distribution of ADP-ribosylated XLas across the velocity gradient (Fig. 3b) indicated that the major proportion of XLas was found in the fractions that also contained TGN membranes, as defined by [³⁵S]sulphate pulse-

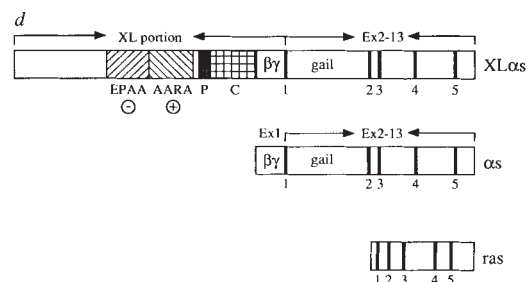
labelled secretogranin II (ref. 14), and that only a minor proportion was present in the top fractions of the gradient, which have been shown to contain constitutive secretory vesicles, plasma membrane, early endosomal membranes and synaptic-like microvesicles¹⁴⁻¹⁶. This was in contrast to the distribution of α s, which was predominantly found in the top fractions of the velocity gradient and of which only a minor proportion was detected in the TGN-containing fractions, as reported previously⁸. Further analysis of the TGN-containing fractions of the velocity gradient by equilibrium sucrose gradient centrifugation

FIG. 2 Primary structure of XLas. **a**, Northern blots of poly(A)⁺ RNA (exon 1, exon 2) or total RNA (XL) from PC12 cells, hybridized with antisense oligonucleotides for exon 1 of α s, exon 2 of α s and XLas (see c), and the XL portion of XLas (see c). Note the weak signal for XLas mRNA with the exon 2, but not the exon 1, probe and the absence of a signal for α s mRNA with the XLas-specific probe. **b**, Immunoprecipitation of XLas with an XLas-specific antibody. Membrane proteins of PC12 cells were [³²P]ADP-ribosylated with cholera toxin and either not immunoprecipitated (total), or immunoprecipitated with the antibody against the C-terminal decapeptide of α s (C-term) or an antibody against a synthetic 12-mer peptide of the XL portion of XLas (see c), in the absence (XL, -) or the presence (XL, +) of the peptide. **c**, Nucleotide and deduced amino-acid sequence of XLas. The arrow at nucleotide 1,541 indicates the beginning of exon 2 of α s. Brackets above the nucleotide sequence indicate the position of the antisense XL oligonucleotide used for northern blotting (a) (nucleotides 1,331-1,350), the sense and antisense oligonucleotides used for the *in situ* hybridization (Fig. 4) (nucleotides 1,444-1,488) and that of the exon 2 antisense oligonucleotide used for northern blotting and screening of the cDNA library (nucleotides 1,583-1,603). Lines below the nucleotide sequence indicate polyadenylation signals. Brackets below the amino-acid sequence indicate the peptides used to raise the XLas-specific antiserum (amino acids 453-464), the exon 2-specific antiserum (amino acids 499-512) and the antiserum against the C terminus (amino acids 837-846), the latter two antisera recognizing both XLas and α s. Lines below the amino-acid sequence indicate the EPAA and AARA repeats, asterisks mark prolines/serines and arginines spaced at six-residue intervals. Arrows beginning at amino-acids 168 and 328 indicate the beginning and end of the alanine-rich domain, arrowheads at residues 336 and 359 that of the proline-rich hinge domain. Triangles indicate the cysteines in the cysteine-rich domain. Nucleotide 1,994 is C rather than T (ref. 9), resulting in a Leu→Pro substitution; this presumably reflects a point mutation in PC12 cells. **d**, Putative domain organization of XLas in comparison with that of α s and ras. Light shaded box (residues 1-167): acidic and polar N-terminal domain (19% Glu/Asp, 18% Ser/Thr, 14% Pro, 7% Arg/Lys). Oblique hatched boxes (residues 168-328): alanine-rich repetitive domain (45% Ala, 13% Pro) containing 9 EPAA repeats (residues 168-235, all charged amino acids are acidic (19% Glu, 3% Asp)) and six AARA repeats (residues 234-328, all charged amino acids (except Glu 244) are basic (12% Arg)); between residues 197 and 269, every sixth amino acid is proline or serine (turn-inducing), and between residues 299 and 323, every sixth amino acid is arginine. Dark shaded box (P, residues 336-359): proline-rich hinge domain (42% Pro) containing a homology to the putative Golgi localization signal of sGai2 (ref. 27). Crossed shaded box (C, residues 360-452): cysteine-rich intermediate domain (contains 6 of the 7 cysteines of the XL portion, 17% Ser/Thr, 16% Arg/Lys, 14% Glu/Asp). $\beta\gamma$ (residues 453-498): exon 1 equivalent of XLas with homology to the $\beta\gamma$ binding site of α s (see e). Vertical black bars numbered 1-5: GTP binding sites²⁸; gail, G α insert or postulated GAP-like domain of α s²⁹. **e**, Comparison of the C-terminal amino-acid sequence of the XL portion of XLas (residues 453-498) with the corresponding N-terminal sequences of various G α subunits. Vertical lines between the XLas and the α s sequence indicate identical or highly homologous residues. Boxes indicate residues that are conserved between most G α subunits and XLas. The Ser/Asn residues corresponding to position 475 of XLas have been included because of their similar turn-inducing potential. When at a given position in the G α subunits the presence of a charged residue is conserved, this is also the case for the corresponding position in XLas (such as Lys 473, Glu 484). Residues 467-468 of XLas are not acidic, in contrast to the corresponding positions in most G α subunits. For α 12, α 13 and α 15, the very N-terminal sequence is not shown. *at*, Transducin; *ag*, gustducin; *r*, rat; *m*, mouse.



METHODS. Standard techniques of molecular biology were used³⁰. **a**, Total RNA and poly(A)⁺ RNA were prepared from PC12 cells and analysed by northern blotting using the oligonucleotides 5'-GGCCCGGTAG-ACCTGCTT-3' for exon 1 of α s, 5'-ACCCATTACATGTAGGATCC-3' (see c) for exon 2 of α s and XLas, and 5'-ACACTCAGATAGACCGAAGC-3' (see c) for the XL portion of XLas. **b**, [³²P]ADP-ribosylation of membrane proteins and immunoprecipitation were done as described in the legend to Fig. 1, except that the final volume in the immunoprecipitation reaction was 400 μ l and 7 μ l of the C-terminal antibody and 14 μ l of the XLas-specific antibody were used. The competing peptide was added to a concentration of 5 μ g ml⁻¹. The XLas-specific antibody was raised in a rabbit by standard techniques after glutaraldehyde-coupling of the peptide RRKQMRKEAMEM (see c) to BSA. **c**, A CDM8 cDNA library (a gift from R. Edwards, UCLA) prepared from PC12 cell mRNA was screened with the exon 2 oligonucleotide of α s, and positive clones were rescreened with the oligonucleotide specific for exon 1 of α s. Clones that were negative in the latter screen were analysed by restriction digests. The clone with the biggest insert, CDM8-XL (nucleotides 380-2,956), was sequenced by conventional and automated methods in both directions (XL portion) or one direction (α s-portion). GC-rich regions were sequenced as described³¹. RACE was done using the Clontech kit, following the instructions of the manufacturer. Briefly, 3 μ g total PC12 RNA were reverse transcribed using an oligonucleotide from the 5'-end of the CDM8-XL insert as a primer. The AmpliFINDER Anchor was ligated to the single-stranded cDNA followed by PCR amplification using the AmpliFINDER anchor primer and a nested XLas oligonucleotide. PCR products (RACE I.2, nucleotides 351-532; RACE I.24, nucleotides 164-532) were cloned into the Invitrogen pCRTMII vector and sequenced in both directions. This protocol was repeated twice, now using oligonucleotides corresponding to the 5'-end of RACE I.24 and RACE II.7 as primers to obtain RACE II.7 (nucleotides 35-269) and RACE III.1 (nucleotides 1-77), respectively. A genomic clone (nucleotides 4-269) was obtained by PCR, using oligonucleotides from RACE III.1 and RACE II.7 as primers and genomic PC12 cell DNA as template.

C	
1	CGCGAGGCTGTGAGATCTCTAACTTCGATACGACATTTCCTCCCAATGGAGATCACCAGA
61	CCCCTGCTTGAGATTGGCAGACGCTCCACTGGGTCGACGACACCGCTGTCAATATG
121	GACAGCCCCCAATCGCAGTGTGCGCCGCTTGAAGTCTCGGAGGCGGCAAGTAA
181	AGCGAGCAGCAAGAGACCCCACTTGAAGCGCAAGCAGCGAGACAGGAACAGCCCT
241	ATCAGCAGCCCACTGCGAGGAGGCAAAAGTCCCTCCCTCGAGCGAGGAGAGATCC
301	CCCACCAACCTGAAGACAGTATCAAGCCAGCTCTGTTCGGAGTCCGGAACGGAC
361	TCCTCAAAGCGATCGGAGTACGCTACACAGCAGTCTTCTGAGTCCGCTGAGGAA
421	GTGCGAGGATCCCAACCATGCCACCGATCTTCCGCTGCTTCTGAAGATCGCGCCCA
481	GATGTCGGGAGCAACAGACGGAGGAGCAGCCCGAGCCCGCTGCGGAGTCCGAAGAC
541	AACAGAGAACGAGCGCGCGCGCGCGCGGAGCGCGCGCGAGCGCGCTGAGCCA
601	GCGCGGAGCG
661	GAGCGAGTCTGCTCCGCGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
721	GCCTCTGCGCAGCG
781	GCTACCCGGGCTGCTCTTCTGCCAGAGCCATCCAGCGCGCGCGCGCGCGCGCGCG
841	TCAGCGATGTGAGCGGCTGCTAGGCGAGCGCGCGCTAGAGCAGCCTATGCGAGTCTCTG
901	GTCTGGGAGCGAGTCTCTCTGCTACTCCAGCGCGCTCGGCGATCCCTCCCTGCCGCG
961	GCAGCAGTCTGCGCGCGCGAGCAGCTCTGCTGCGCGCGCGCTGCGCGCGCGCGCGCG
1021	TCTGCGCGCGCGAGCG
1081	CCGCTACTCCGCGCGCGCTGCTCCGCGCGCGAGTCCCTGGCTGACAAATACGAAGTGGC
1141	CGAAGCTGCTGAGGATGAGGCTGCGTCCGCGATCGGAGATCGAGTCTCCAGCGAT
1201	GAGTCCGAAGAGGGGCGCTGCTGCTTCCAGTGGCTTTCGCGCGGAAACCGCGCGCT
1261	GGCCAGCGCGCGGAGCGCACAGTCCGAGGCAACCGCGCGCGCGCGCGCGCGCGCG
1321	TTGGAAGTCTGCTCGGCTTCTGAGTATCCGATCAGATCCCTCAGCGCGCGGAGG
1381	GCCAAGGATCTTGAAGAGAGCGCAACAGATGCGCAAGAGCGCTGAGGATCGCA
1441	GAGCAGAGGCGCGCAGATAAGAAACGCGAGCTCATCGACAGCACTGGAGGAGGAG
1501	AAGATGACATACATGTGTACACCGCGCTGCTGCTTCTAGTGTGAGAGTCTGGCAAA
1561	AGCACCATTGTGAAGCAGATGAGTCTTAACTGAGTTTAAAGGAGGCGCGCGCG
1621	GAAGAGGACCGCGAGGCTGCAAGGAGCAACAGCGATGTTGAGAGGCGCAAGTGCAG
1681	GACATCAAAACCACTGAAGGAGGCGCTTGAACCACTTGTGCGCGCATGAGCAACCTG
1741	GTGCCCCCGTGGAGCTGCGCAACCTGAGAACCGTTCAGAGTGGATACATCTGAGC
1801	GTGATGAACGTGCCAACTTGTACTTCCCACTGAATTTCTATGAGCATGCCAAGCTCTG
1861	TGGGAGGATGAGGAGTCTGCTGCTGCTACGAGCGCTCCACAGTACAGCTGATCGAC
1921	TGTGCCAGTACTTCTGCGCAAGATTGATGTATCAAGAGCGCGCTACGTGCCAAGT
1981	GACCAGGACCTGCTGCTGCGCGCGCTGCTGACCTTCTGGAATCTTTGAGCAAGTTCAG
2041	GTGGCAAACTCACTTCCACATGTTGATGTGGCGCGCGCGCGATGAACCGCGCAG
2101	TGGATCCAGTGCTTCAATGATGTGATGCTCATCTTCTGTTGTTGCCAGCAGCAGTAC
2161	AACATGCTACTCCGGGAGGACCAACAGACCAACCGTCTGAGGAGGCTTGAACCTCTTC
2221	AAGAGCATCTGGAACACAGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
2281	CAAGATCTGCTTGTGAGAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
2341	GAGTTCGCTGCTACACCACTCTGAGGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
2401	GTGACCGCGCGCAAGTACTTCTATCCGGATGAGTTTCTGAGATCAGCACTGCTAGTGA
2461	GATGGAGCTGCTACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
2521	CGTGTCTTCAAGACTGCGGTGACATCATCCAGCGCATGCTTCTGCCAATACGAGCTG
2581	CTCTAAGAGGGAACGCCCAATTTAATTCAGCCTTAAGCACAATTAAATAGAGTGAAA
2641	CGCAATCGTACAAGCAGTGTGATCACCCACCATAGGCGATGATCAACACCGCAACCTTTC
2701	CTTTCTCCCGAGTATCTGAAACCGCCCTCTCCCTCAGCTGCTGCTGCTGCTGCTGCTG
2761	ATTAGTAAGCTTAAGCGCGCTACAGAAGAAAAAGAAAAAGGCAACAAAGTT
2821	CCCTCTCACTTTCAGTAAATAAATAAAGCAGCAACAAACAGAAATAAAGAAATAAATG
2881	AAATAAATGAAACTCAAAATGAATAAATATTGTGTTGTGAGCATTAAAAATCAAAAT
2941	AAAAATTAACATTTAG 2956



e	
453	R R K Q M R K E A M E M R E Q K R A D K K R S K L I D K Q L E E E K M D Y M C T H R L L L L G XLαs r
460	M G C L G N S K T E D Q R N E E K A Q R E A N K I E K Q L Q K D K Q V Y R A T H R L L L L G αs r
470	M G C . T V S A E D K A A A E R S K M I D K N L R E D G E K A A R E V K L L L L G αi2 r
480	M G C . T L S A E E R A A A L E R S K A I E K N L K E D G I S A A K D V K L L L L G αo1 r
490	M G A . G A S A E E K H S R E L E K K L K E D A E K D A R T V K L L L L G αt m
499	M G S . G I S S E S K E S A K R S K E L E K K L Q E D A E R D A R T V K L L L L G αg r
	M T L E S M M A C C L S D E V K E S K R I N A E I E K Q L R R D K R D A R R E L K L L L L G α11 m
	E A G A R E R R A G A A R D A E R E A R R R S R D I D A L L A R E R R A V R R L V K I L L L L G α12 m
	S V L S V C F P G C V L T N G E A E Q Q R K S K E I D K C L S R E K T Y V K R L V K I L L L L G α13 m
	M T L E S I M A C C L S E E A K E A R R I N D E I E R H V R R D K R D A R R E L K L L L L G αq m
	M G C R Q S S E E K E A A R R S R R I D R H L R S E S Q R Q R R E I K L L L L G αz r
	R S L T W G C C P W C L T E E E K T A A R I D Q E I N R I L L E Q K K Q E R E E L K L L L L G α15 m

gation (Fig. 3c, d) revealed the cofractionation of XLas with the [35 S]sulphate pulse-labelled secretogranin II, showing that it is indeed a membrane protein of the TGN.

By *in situ* hybridization of various rat tissues using probes specific to the XL portion of XLas (Fig. 4), XLas mRNA was not detected in liver and kidney. In the adrenal gland, XLas mRNA was found in the central region containing the medullary chromaffin cells, but not in the cortex. In the pituitary gland, XLas mRNA was abundant in cells of the intermediate lobe

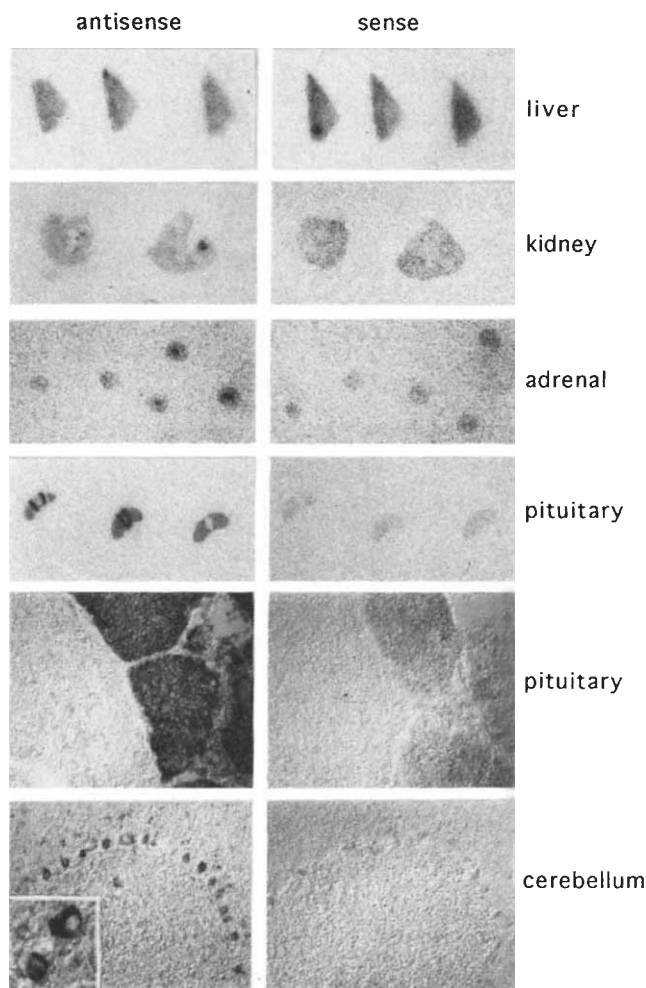


FIG. 4 XLas is only found in cells containing both the regulated and constitutive pathway of protein secretion. *In situ* hybridization of cryosections of rat liver, kidney, adrenal and pituitary using 32 P-labelled oligonucleotides (top 8 panels), and pituitary and cerebellum using digoxigenin-labelled riboprobes (bottom 4 panels). For the *in situ* hybridizations using 32 P-labelled oligonucleotides, the multiple sections shown represent consecutive cryosections which were alternately hybridized with either the antisense or the sense probe. The inset in the bottom left panel shows two Purkinje cell bodies at higher magnification. METHODS. Organs were dissected from adult rats and fixed for 3–5 h at 4 °C by immersion in 4% paraformaldehyde/PBS, pH 7.5. Cryosections of 10 μ m were prepared, collected onto polylysine-coated glass slides and further fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. *In situ* hybridization using radioactive probes was as described³³, using 45-mer sense and antisense oligonucleotides (2 ng ml⁻¹) specific for the XL portion of XLas (for sequence, see Fig. 2c) that had been labelled with terminal transferase and [32 P]dCTP. Sections were subjected to autoradiography using X-ray film. The procedure for *in situ* hybridization using digoxigenin-labelled sense and antisense riboprobes (RACE I.24, see legend to Fig. 2c) was modified from that used for whole-mount preparations³⁴ and that used for paraffin sections³⁵, as will be described in detail elsewhere (J.M. and W.B.H., manuscript in preparation).

and well above background in cells of the anterior lobe. XLas mRNA was not detected in the neurohypophysis, which besides the axon terminals of hypothalamic neurons contains special glial cells (pituicytes). Finally, in the cerebellum, a strong signal for XLas mRNA was observed in the perikarya of Purkinje cells. By northern blot analysis, the XLas mRNA was detected in adrenal gland, brain and pituitary, but not in liver and kidney (data not shown). Using *in vitro* ADP-ribosylation catalysed by cholera toxin followed by immunoprecipitation of membrane proteins with the antibody against the C terminus of α s, XLas was found in AR42J cells, a rat pancreatic cell line with both exocrine and neuroendocrine secretory granules (O. Bräunling and W.B.H., unpublished observations), but not in NIH-3T3, LMTK⁻ and RK13 cells (data not shown). These results suggest that XLas occurs predominantly, if not exclusively, in cells containing both the regulated and the constitutive pathway of protein secretion.

In conclusion, XLas is a new type of GTP-binding protein in which the functional domains of a $G\alpha$ subunit are linked to a large new protein structure (Fig. 2d). Our data have several implications. First, the selective occurrence of XLas in certain populations of endocrine cells and neurons, and its specific association with the TGN, suggest that XLas has a role in a TGN function that is characteristic of these cells, such as the formation of two types of secretory vesicles. The stimulation of secretory vesicle formation by cholera toxin⁸ may well be mediated by XLas. Second, some of the pathological consequences of mutations in the exon 2–13 portion of the α s gene^{17,18}, especially those concerning neuroendocrine tissues, may be due to an alteration in the function of XLas. Third, our data raise the question whether other XL α proteins exist, that is, proteins in which the functional domains of $G\alpha$ subunits are linked to specific XL portions. Interestingly, proteins of ~100K that are recognized by antibodies against the C-terminal decapeptides of α s and α t/ α i and that are clearly distinct from XLas have been reported^{19,10}. One of these binds GTP and is associated with endosomal membranes^{19,22}. Perhaps 'extra large' (≥ 90 K) $G\alpha$ -subunit-related GTP-binding proteins, associated with specific intracellular compartments, are a new class of regulatory proteins. □

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Localization of pre-mRNA splicing in mammalian nuclei

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In mammalian nuclei, precursor messenger RNA splicing factors are distributed non-uniformly. Antibodies directed against structural polypeptides of small nuclear ribonucleoprotein particles (snRNPs)¹ and some non-snRNP splicing factors² have shown that these components are concentrated in about 20–50 nuclear ‘speckles’. These and other non-homogeneous distributions have been proposed to indicate nuclear ‘compartments’ that are distinct from the sites of transcription and in which RNA processing occurs^{3–9}. We have tested this idea using a new approach. Previous

structural^{10–13} and biochemical^{14–16} data have shown that splicing can occur in association with transcription. Nascent RNA of specific genes can be detected by *in situ* hybridization as intense spots of nuclear stain which map to the sites of transcription^{17–19}. Here we identify active pre-mRNA splicing sites by localizing the nascent spliced mRNA of specific genes. We find that splicing occurs at the sites of transcription, which are not coincident with intranuclear speckles. We conclude that the nucleus is not compartmentalized with respect to transcription and pre-mRNA splicing.

Initially, we analysed the well-characterized adenovirus type 2 (Ad2) major late pre-mRNA. HeLa cells were infected with Ad2, giving rise to a collection of identical, randomly distributed transcription units. Viral nucleic acids were localized by one-step *in situ* hybridization using fluorochrome-labelled oligonucleotide probes that were complementary to an untranscribed region of Ad2 DNA (DNA probe), an Ad2 intron (intron probe) or a splice-junction²⁰ (splice-junction probe) (Fig. 1, bottom; see legend for details). To avoid detecting signals of replicating viral DNA, Ad2-infected HeLa cells were treated with the DNA-synthesis inhibitor cytosine β -D-arabino-furanoside.

Figure 1 examines by digital imaging microscopy the collection of intranuclear signals obtained using the Ad2 DNA probe (Fig. 1a), intron probe (Fig. 1b), or splice-junction probe (Fig. 1c). Each of the probes gave rise to a similar pattern of about twenty intranuclear punctate signals. The signals were specific for Ad2 nucleic acids because no hybridization signals were observed in uninfected HeLa cells (d–f). The additional control experiments in Fig. 1 (g–l) demonstrate that the DNA, intron and splice-junction probes detected specifically Ad2 DNA, intron-containing Ad2 RNA and spliced Ad2 mRNA, respectively. Furthermore, all of the results in Figs 1 and 2 (see below) support the notion that each signal represents an Ad2 major late transcription unit.

To define the spatial relationship between transcription and splicing, we used pairwise combinations of the three probes using two-colour fluorophores (green and red). Cells were examined by digital imaging microscopy, allowing the images to be over-

FIG. 1 *In situ* detection of Ad2 nucleic acids in virally infected cells. *In situ* hybridization was used to detect Ad2 nucleic acids in HeLa cells using specific probes for DNA (a, d, g, j), an intron (IN; b, e, h, k), a splice-junction (SJ; c, f, i), or a splice-junction control probe (SJ-C; l). Cells were treated with 100 $\mu\text{g ml}^{-1}$ RNase (j, h, k, l) or 5 U per 30 μl DNase (g, k) before hybridization. A diagram of the probes is also shown: boxes, exons; lines, introns.

METHODS. Oligonucleotides containing amino-modified thymidine (Glen Research) were synthesized (Model 394, Applied Biosystems), purified (on a 10% polyacrylamide gel) and labelled with fluorescein isothiocyanate (FITC) (Molecular Probes) or CY3 (BRL) in 0.1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (pH 9.0) overnight (100-fold excess), and further purified through a Sephadex-G50 column and a 10% polyacrylamide gel. Probes: Ad2 splice-junction probe, 5'-CTCAACCGCGAG/CCCAACAGCTGG-3', complementary to the splice-junction sequence of the major late transcription unit, first and second leaders (6,066–6,077/7,099–7,110); the SJ-C probe was complementary to nucleotides 7,099–7,110/6,066–6,077; Ad2 intron probe, 5'-TTGTCTTTCTGACAGATGGACG-3', complementary to intron sequence of 6,192–6,215; two Ad2 DNA-specific probes, 5'-GTGTTCCGCCACACTGCAACATC-3' and 5'-AATACACTGCGCGTCAGCT-GACTA-3', complementary to non-transcribed 5' DNA regions (121–144, 448–471). *In situ* hybridization: HeLa cells grown on glass coverslips were infected with Ad2 for 1 h at 100–200 PFU per cell in cytosine β -D-arabino-furanoside (araC, 20 $\mu\text{g ml}^{-1}$), grown for 18–24 h, fixed (in 4% paraformaldehyde for 15 min) and stored (in 70% ethanol at 4 °C), rehydrated in PBS and 2 $\times$ SSC. 20 μl hybridization solution (20 ng probes, 20% formamide, 2 $\times$ SSC, 10% dextran sulphate, 1% BSA, 10 $\mu\text{g tRNA}$, 10 μg single-stranded DNA) was added onto each coverslip, sealed with parafilm, and incubated for 2 h at 37 °C. For DNA probes, hybridization pretreatment of cells was at 70 °C in 70% formamide and 2 $\times$ SSC for 2 min. Washing was for 15 min each in 2 $\times$ SSC/20% formamide, 1 $\times$ SSC/20% formamide, 1 $\times$ SSC, PBS. Cells were examined by digital imaging microscopy after mounting in glycerol (Fig. 2 legend).

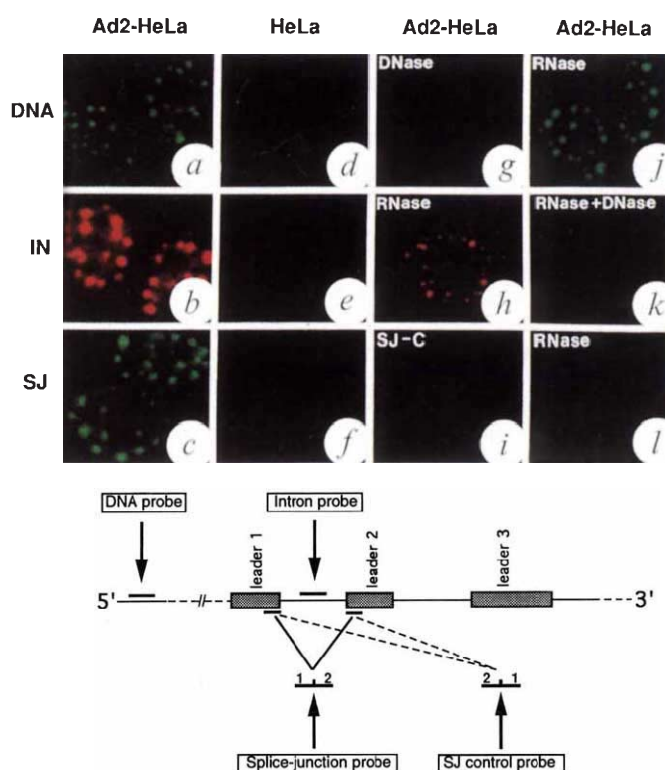
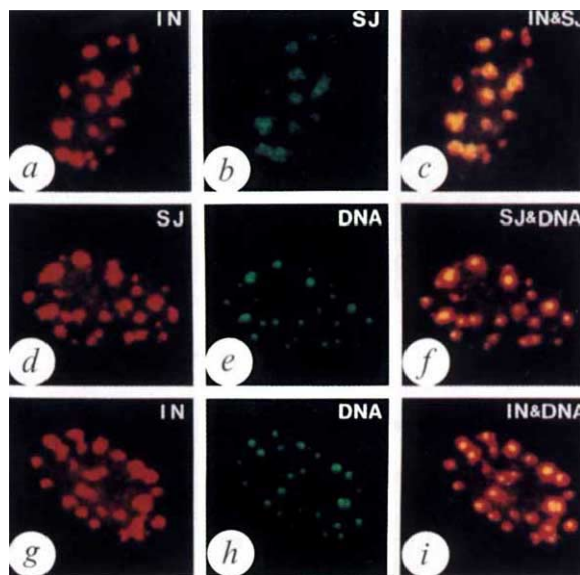


FIG. 2 Spatial relationship of Ad2 DNA and RNA signals. Spatial relationship of Ad2 spliced RNA, intronic RNA and Ad2 DNA examined by pairwise combinations using two-colour fluorophores: *a*, *b* and *c*, congruence of intronic Ad2 RNA (IN) relative to spliced Ad2 RNA (SJ); *d*, *e* and *f*, congruence of spliced Ad2 RNA (SJ) relative to Ad2 DNA (DNA); *g*, *h* and *i*, congruence of intronic Ad2 RNA relative to Ad2 DNA; *c*, *f* and *i*, alignment of green and red appears yellow.

METHODS. *In situ* hybridization is described in Fig. 1 legend. Images of cells were captured using a cooled charge-coupled device (CCD) camera, digitized and analysed³⁰. Green and red pseudo-coloured images from the same optical plane were superimposed using dual-coloured fluorescence beads as fiducial markers.



laid with precise alignment. Alignment of the green and red signals results in yellow. Figure 2 shows that the signals detected with the DNA, intron and splice-junction probes co-localized, allowing several conclusions to be drawn. First, within the resolution of the light microscope, spliced Ad2 mRNA accumulated directly at the sites of Ad2 transcription. Second, the co-localization of the signals detected by the DNA and RNA probes indicates that no viral genome was located in a nuclear region that could not support transcription. Finally, the co-localization of the signals detected by the intron and splice-junction probes indicates that no viral transcription unit was located in a nuclear region that could not support splicing.

The spatial relationship of the Ad2 pre-mRNA splicing sites to the intranuclear speckles was visualized with the splice-junction probe (SJ in Fig. 3*A*). These same cells were stained by immunofluorescence using an anti-Sm antibody (Fig. 3*A*, *a-c*) which recognizes a component of snRNPs; an anti-3C5 antibody (Fig. 3*A*, *d-f*) which recognizes a family of conserved structural proteins present in the intranuclear speckles²¹; or an anti-SC35 antibody (Fig. 3*A*, *g-i*), which recognizes the essential splicing factor SC35². With the anti-Sm and anti-3C5 antibodies, the sites of Ad2 pre-mRNA splicing appeared to be randomly distributed throughout the nucleoplasm and did not co-localize with intranuclear speckles (Fig. 3*A*, *c* and *f*). In contrast, the anti-SC35 antibody (Fig. 3*A*, *g-i*) indicated co-localization of Ad2 splicing sites with sites of concentrated SC35 (Fig. 3*A*, *i*).

To investigate why the results with the anti-SC35 antibody were anomalous, we compared the patterns detected with the three antibodies in uninfected and Ad2-infected HeLa cells. Figure 3*B* shows that in uninfected HeLa cells the signals detected by all three antibodies are co-localized. In contrast, following Ad2 infection (Fig. 3*C*), Sm and 3C5 antigen distributions are still co-localized (Fig. 3*C*, *g-i*), whereas the SC35 antigens no longer co-localized with Sm (Fig. 3*C*, *a-c*) and 3C5 (Fig. 3*C*, *d-f*) antigens.

We considered that the results with anti-SC35 antibody were due to cross-reaction with an Ad2 protein(s). Figure 3*D* shows that in an extract prepared from Ad2-infected cells, in addition to the expected SC35 band of *M_r* 35,000 (35K; lane 1), a major 72K and a weaker 44K polypeptide were also detected (lane 2). Immunoblotting with an antibody directed against the Ad2 DNA-binding protein (DBP) demonstrated that the 72K and 44K polypeptides corresponded to intact Ad2 DBP and its C-

terminal proteolytic fragment²² (DBP*, lane 4). Consistent with this, Fig. 3*E* shows that in Ad2-infected cells SC35 co-localized with Ad2 DBP (Fig. 3*E*, *a-c*). As expected, DBP also co-localized with Ad2 DNA (Fig. 3*E*, *d-f*), the site of Ad2 transcription and splicing (Fig. 2). Thus, the apparent co-localization of SC35 and Ad2 splicing sites in Fig. 3*A* was due to cross-reaction of the anti-SC35 antibody with the Ad2 DBP (see also ref. 23).

Because our results were obtained with Ad2, an unintegrated viral genome, we analysed an endogenous cellular gene, the β -actin gene. Figure 4*A* shows that the β -actin-specific intron (Fig. 4*A*, *a*) or splice-junction (Fig. 4*A*, *b*) probe revealed two nuclear spots that co-localized (Fig. 4*A*, *c*). As expected, these RNA signals were coincident with the β -actin gene (data not shown). Figure 4*B* maps the β -actin signals detected with the splice-junction probe relative to the speckles. The results indicate that in most cases the nascent spliced β -actin RNA did not coincide with a nuclear speckle. In some cases, even within a single cell, one of the actively transcribed β -actin genes co-localized with a speckle whereas the other did not.

The spatial organization within the nucleus of pre-mRNA processing is controversial. We have shown that splicing of viral and cellular pre-mRNAs takes place spatially and temporally at the sites of these genes. There was only a random correlation between the sites of transcription and the nuclear speckled domains. Our results complement previous studies indicating that transcription occurs throughout the nucleus, with no evidence for compartmentalization²⁴⁻²⁶. Although our results show that different regions of the nucleus can support transcription and pre-mRNA splicing, we cannot yet exclude the speckles as processing sites for some pre-mRNAs.

In previous work, which also used adenovirus as a model, it was concluded that cellular splicing components move from the speckles to the sites of viral transcription²⁷. In contrast, we find that the intranuclear distribution of speckles is unaffected by viral transcription, which occurs randomly throughout the nucleus. This discrepancy in our results may be explained as the previous work relied heavily upon the anti-SC35 antibody as a marker for speckles, which we have shown cross-reacts with Ad DBP (Fig. 3); also, inhibitors of viral DNA replication were not used²⁷, and the resulting high levels of viral nucleic acids may have perturbed the normal distribution of cellular splicing factors.

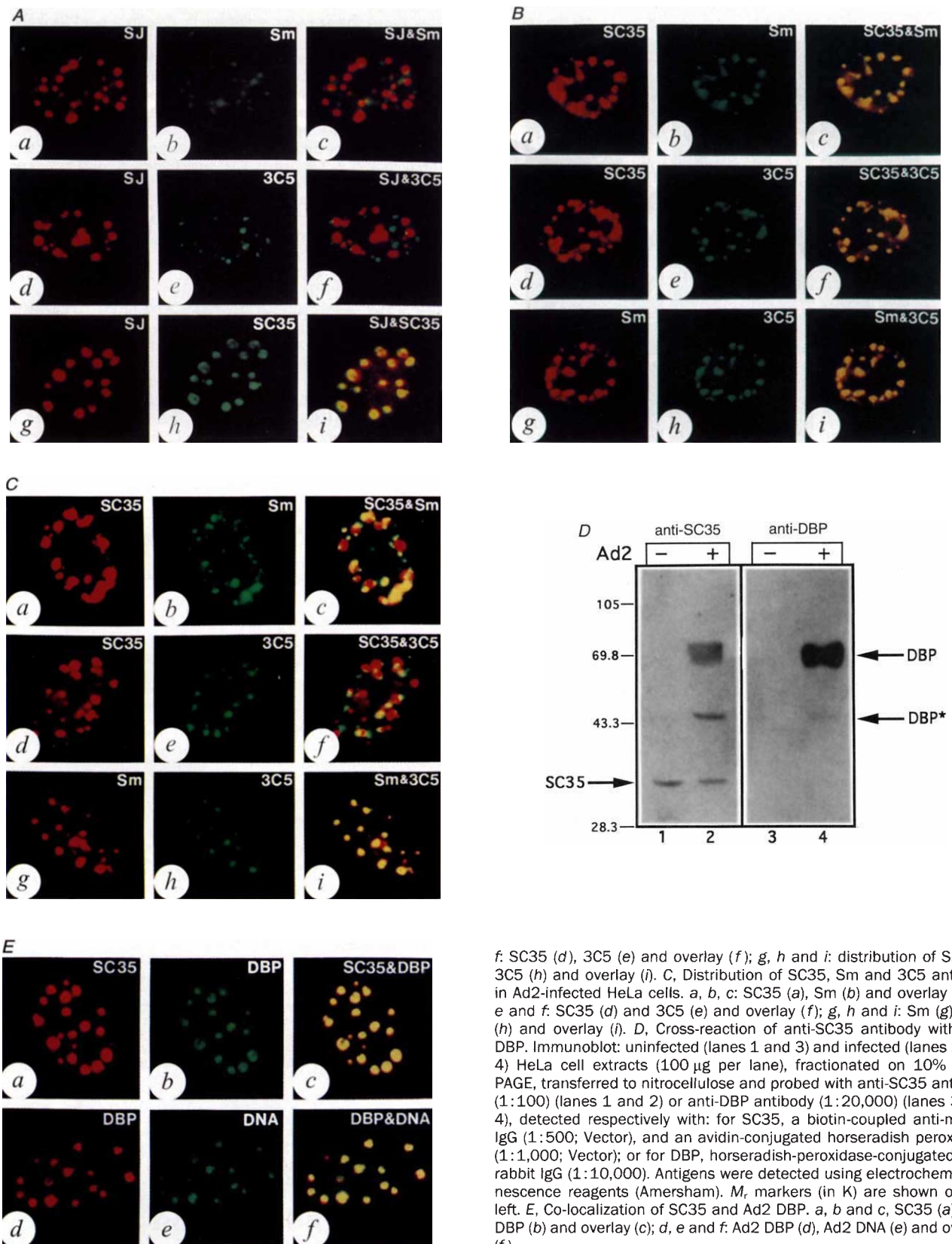


FIG. 3 Localizing sites of Ad2 pre-mRNA splicing. **A**, Spatial correlation of Ad2 splicing sites relative to intranuclear speckles. Distributions examined: *in situ* hybridization using the splice-junction probe (**a**, **d**, **g**) with immunofluorescence using anti-Sm (**b**), anti-3C5 (**e**) or anti-SC35 (**h**) antibodies. **B**, Distribution of SC35, Sm and 3C5 antigens in uninfected HeLa cells. **a**, **b** and **c**: SC35 (**a**), Sm (**b**) and overlay (**c**); **d**, **e** and **f**: SC35 (**d**) and 3C5 (**e**) and overlay (**f**); **g**, **h** and **i**: Sm (**g**), 3C5 (**h**) and overlay (**i**). **C**, Distribution of SC35, Sm and 3C5 antigens in Ad2-infected HeLa cells. **a**, **b** and **c**: SC35 (**a**), Sm (**b**) and overlay (**c**); **d**, **e** and **f**: SC35 (**d**) and 3C5 (**e**) and overlay (**f**); **g**, **h** and **i**: Sm (**g**), 3C5 (**h**) and overlay (**i**). **D**, Cross-reaction of anti-SC35 antibody with Ad2 DBP. Immunoblot: uninfected (lanes 1 and 3) and infected (lanes 2 and 4) HeLa cell extracts (100 µg per lane), fractionated on 10% SDS-PAGE, transferred to nitrocellulose and probed with anti-SC35 antibody (1:100) (lanes 1 and 2) or anti-DBP antibody (1:20,000) (lanes 3 and 4), detected respectively with: for SC35, a biotin-coupled anti-mouse IgG (1:500; Vector), and an avidin-conjugated horseradish peroxidase (1:1,000; Vector); or for DBP, horseradish-peroxidase-conjugated anti-rabbit IgG (1:10,000). Antigens were detected using electrochemiluminescence reagents (Amersham). **M**, markers (in K) are shown on the left. **E**, Co-localization of SC35 and Ad2 DBP. **a**, **b** and **c**: SC35 (**a**), Ad2 DBP (**b**) and overlay (**c**); **d**, **e** and **f**: Ad2 DBP (**d**), Ad2 DNA (**e**) and overlay (**f**).

f: SC35 (**d**), 3C5 (**e**) and overlay (**f**); **g**, **h** and **i**: distribution of Sm (**g**), 3C5 (**h**) and overlay (**i**). **C**, Distribution of SC35, Sm and 3C5 antigens in Ad2-infected HeLa cells. **a**, **b**, **c**: SC35 (**a**), Sm (**b**) and overlay (**c**); **d**, **e** and **f**: SC35 (**d**) and 3C5 (**e**) and overlay (**f**); **g**, **h** and **i**: Sm (**g**), 3C5 (**h**) and overlay (**i**). **D**, Cross-reaction of anti-SC35 antibody with Ad2 DBP. Immunoblot: uninfected (lanes 1 and 3) and infected (lanes 2 and 4) HeLa cell extracts (100 µg per lane), fractionated on 10% SDS-PAGE, transferred to nitrocellulose and probed with anti-SC35 antibody (1:100) (lanes 1 and 2) or anti-DBP antibody (1:20,000) (lanes 3 and 4), detected respectively with: for SC35, a biotin-coupled anti-mouse IgG (1:500; Vector), and an avidin-conjugated horseradish peroxidase (1:1,000; Vector); or for DBP, horseradish-peroxidase-conjugated anti-rabbit IgG (1:10,000). Antigens were detected using electrochemiluminescence reagents (Amersham). **M**, markers (in K) are shown on the left. **E**, Co-localization of SC35 and Ad2 DBP. **a**, **b** and **c**: SC35 (**a**), Ad2 DBP (**b**) and overlay (**c**); **d**, **e** and **f**: Ad2 DBP (**d**), Ad2 DNA (**e**) and overlay (**f**).

METHODS. After hybridization, cells were incubated with primary antibodies (PBS/1% BSA for 1 h), washed (PBS/0.1% BSA for 0.5 h), incubated with secondary antibodies (PBS/1% BSA for 1 h), rinsed and mounted onto glass slides. Antibody dilutions: anti-SC35 and anti-Sm antibodies, 1:100; anti-3C5 IgM, 1:4; anti-Sm (Immunovision), 1:40; FITC and CY3 (both Sigma) goat anti-mouse IgG secondary antibodies, 1:150 and 1:100, respectively; FITC goat anti-mouse IgG (Sigma), 1:40.

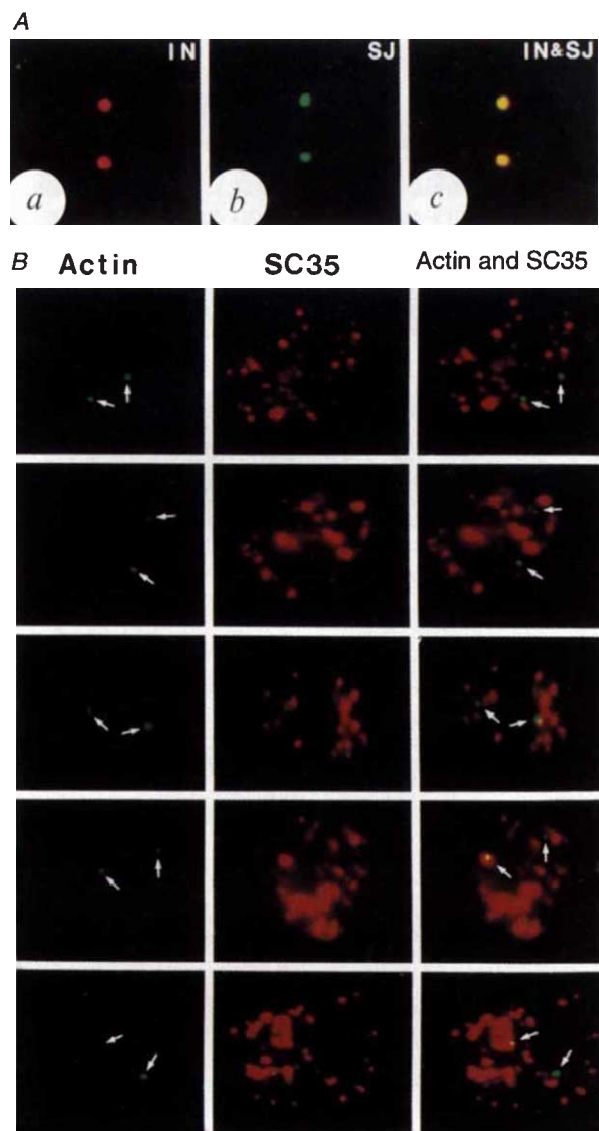


FIG. 4 Localizing sites of endogenous β -actin pre-mRNA splicing. **A**, Spatial correlation of unspliced and spliced β -actin RNAs. *In situ* hybridization signals detected using intron (a) and splice-junction (b) probes. (c), Overlay. **B**, Distribution of β -actin RNA relative to intranuclear speckles. Distributions of actively transcribed β -actin RNA (actin) relative to intranuclear speckles (SC35) in five representative cells. Nuclear β -actin RNA signals are indicated by arrows.

METHODS. Oligonucleotide probes contained 24 nucleotides complementary to actin sequence and 18 nucleotides of nonspecific sequence added to increase the fluorochromes. Intron-specific probes: IN1, 5'-GTCCCTGTGCAGAGAAAGCGCCCT-3'; IN2, 5'-CACGGCTAAGTGTGCTGGGGTCTT-3'; IN3, 5'-ATGAGGGCAGGACTTAGCTTCCAC-3'; IN4, 5'-CTGACCTGCCAGGTCAGCTCAGG-3' complementary to the regions of 1,088–1,111, 1,744–1,768, 2,354–2,377 and 2,587–2,610. Splice-junction probes: SJ1, 5'-CACCATCACGCC/CTGGTGCCTGGG-3'; SJ2, 5'-CTCAAACATGAT/CTGGGTCATCTT-3'; SJ3, 5'-GGACTCCATGCC/CAGGAAGGAGG-3'; and SJ4, 5'-AGGAGCAATGAT/CTTGATCTTCAT-3' complementary to 1,028–1,039/1,074–1,085, 1,401–1,412/1,854–1,865, 2,281–2,292/2,388–2,399 and 2,559–2,570/2,683–2,694. Nonspecific sequences were: 5'-TTGCTTGCTTGCTTGCTT-3'. Morphometric analysis of nuclei was done on photographic images of optical sections from ten representative cells using a combined Image-Lab and Image-Measure program (Micro-Science, Inc.). The speckles occupied $31 \pm 7\%$ of the nucleoplasmic area (excluding nucleoli). Of ten β -actin RNA signals, three coincided with nuclear speckles and one was in contact.

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ERRATUM

Signal transduction and regulation in smooth muscle

Andrew P. Somlyo & Avril V. Somlyo

Nature **372**, 231–236 (1994)

IN the UK edition of *Nature*, Figs 1 and 2 of this Review Article were accidentally transposed during the production process. □

What then is the function of nuclear speckles? They could be sites for storage of splicing factors, or more simply sites where excess splicing factors accumulate, as indicated in a study of *Drosophila* polytene nuclei²⁸. It has been shown in yeast that splicing factors are present in large functional excess²⁹.

The approach described here can be extended to other genes in order to provide high spatial and temporal resolution of the transcription and processing of its pre-mRNA. The precise targeting of fluorochrome-labelled oligonucleotide probes to different regions of a large transcript, and the exact superimposition of these images by digital imaging microscopy, will allow a correlation of biochemical and structural events in the early life of an mRNA. □

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Patents in the public interest

Gary Moss and Simon Cohen

The UK High Court has granted an injunction allowing Chiron Corporation a monopoly in selling hepatitis C virus test kits. The decision will be welcomed by the pharmaceutical and biotechnology industries.

LAST month, the UK High Court rejected applications by Murex Diagnostics Limited and Organon Teknika Limited to end Chiron's monopoly in making and selling hepatitis C virus (HCV) test kits (see *Nature* 372, 485; 487; 8 December 1994). The crux of Murex and Organon's case was that an injunction would be contrary to the public interest: first, it was in the public interest that other test kits should be available, not just Chiron's; and, second, an injunction would impede research and development. This decision follows an earlier judgement in which the High Court found Chiron's patent to be valid and infringed.

Much controversy has surrounded the issue of whether injunctions should be granted in these types of cases. The courts do have discretion to award damages rather than injunctions, either by a lump sum or on a royalty-type basis for the remaining term of the patent. But this discretion is exercised only rarely, and we believe that it was right to award the injunction on this occasion and, indeed, that it can hardly ever be right to refuse injunctions in patent cases.

HCV is widely distributed around the world, with 0.1–2 % people affected in Western Europe and North America, 3% in some Mediterranean countries and up to 6% in some tropical areas. Some estimates suggest 300 million people are affected world-wide. Murex is concerned because it believes that only Chiron and companies that license from Chiron will have access to the UK market for hepatitis-C screening. Hepatitis-C tests cost three to four times more than many other virus tests. Murex has until now supplied the UK's National Health Service with more than a third of its HCV test kits.

The companies are also concerned that the injunctions will suppress further research and development on the virus test that would eventually benefit patients. One example is the development by Murex of an assay for serotyping the virus (there are at least six strains of HCV) which Murex believes to be "a unique epidemiological tool". The assay is about to come into use, but would be suppressed by an injunction. (Chiron is also working on its own assay but has not announced when it will be ready.) But in court last month Murex and Chiron sensibly agreed a "stay" on the injunction pending the appeal on this assay only, because there is no alternative currently available.

Although accepting that an injunction gives the patentee a monopoly, thus providing the right to restrict competition and put prices up, the UK patents judge, Mr Justice Aldous, said that this is inherent in any patent system. If an injunction had not been granted here, it would set an unhelpful precedent for arguments that injunctions should not be granted for most pharmaceutical patents, for example antibiotics or anti-inflammatory drugs.

It is true that the injunction means that the HCV test is likely to be more expensive. Nonetheless, there are many advantages of the patent system, mainly in stimulating technical progress: it encourages research and invention; it induces inventors to disclose discoveries; it offers a reward for the expense of developing inventions to the level at which they are commercially practical; and it provides an inducement to invest capital in new lines of production which might not appear profitable if many competing producers embarked on them simultaneously — particularly relevant to medicinal products.

Additionally, patent laws have always contained safeguards to protect the public against abuse by a patentee of monopoly rights: compulsory licenses are available (after 3 years from the date of the patent) if, for example, there were not sufficient HCV test kits available or if the court considered Murex's test kits made a substantial contribution to the art and its working was "prevented or hindered" by Chiron's patent. Further, there are provisions in the UK Patents Act for Crown use, that is for life-saving drugs, medicines and diagnostic kits to be made available publicly in the United Kingdom without the patentee's permission. The Crown has the power to authorize Murex to sell its own test kits.

Injunctions should be refused only in extreme cases: a vital life-saving medicine or an epidemic that requires urgent treatment which could be given only by the infringing product, not merely because the treatment will cost more because of the patentee's monopoly. With safeguards for the public already in place, everything must be done to strengthen the rights of patent owners and the patent system around the world. Otherwise, potentially crucial discoveries can be lost. For example, in countries such as those of Eastern Europe, where researchers rank among the best in the world, inventions cannot be properly exploited because the necessary

infrastructure for obtaining and asserting patents does not exist.

A move in the right direction was the introduction recently of supplementary protection certificates (SPCs) by the European Commission to extend the protection for patents for pharmaceuticals. The term of a European patent is 20 years from the filing of the application, and the patentee cannot bring an action to restrain infringement until the patent is granted. With delays in granting patents, particularly in areas such as biotechnology where there is so much legislative ambiguity and lengthy clinical trials and regulatory approval are often required before a drug can be marketed, the effective monopoly is often as little as 10 years, and can be 3 or 4 years. In contrast, the patent term in the United States is 17 years from grant, so delays in the application procedure have no impact on the length of effective monopoly. The SPC extends the period of protection initially conferred by a patent, once that patent has expired, to compensate to an extent for the patent life lost in obtaining such approval. The duration of the certificate reflects a compromise between the interests of patentees and those of generic producers, and is therefore equal to the patent life lost between the patent filing date and the date of first marketing approval in the community, minus 5 years, subject to a maximum of 5 years.

The judge dismissed Murex's argument that an injunction would impede research and development on the grounds that "there can be no doubt that the Chiron patent monopoly will in the short term deter some companies from carrying out research and development, but that is inherent in the patent system".

For every successful research project, countless others fail miserably. They all require substantial investment. They must all continue if breakthroughs are to be made at the pace they are required. There must be as much incentive as possible for scientists to be encouraged to innovate. In the words of Mr Justice Aldous, "It is in the public interest that patent monopolies be enforced with the resulting restrictions upon competition that are inherent in the patent system". □

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